EXPERIMENTAL CELL RESEARCH

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THE ULTRASTRUCTURE OF THE CORTICAL GRANULES AND THEIR PRODUCTS IN THE SEA URCHIN EGG AS STUDIED WITH THE ELECTRON MICROSCOPE[1]

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THE cortical granules of the sea urchin egg are particulate components of the cytoplasm formed near the young oocyte nucleus and transferred to the cortex during maturation [27, 44]. According to histochemical staining reactions they are composed of polysaccharides as well as proteins [27, 28]. Some of the first clear demonstrations of this component were those of Harvey in 1911 [12], Runnström in 1928 [36], Hendee in 1931 [13] and Lindahl in 1932 [19]. It was also shown that the cortex of the fertilized egg was practically free from cortical granules. Many different explanations have been given for the mechanism of the breakdown of the cortical granules, Moser 1939 [31], Motomura 1941 [32], Runnström, Monné and Wicklund 1944 and 1946 [45, 46] and Endo 1953 [7]. Their function to form part of the fertilization membrane has been stressed, although there is some controversy concerning the details of this mechanism. Their possible role in the formation of the hyaline layer of the fertilized eggs and substances in the perivitelline space is less clear.

There are a few electron microscope studies which demonstrate the ultrastructure of the cortical granules in eggs of the sea urchin *Arbacia punctulata* in ultra-thin sections [6, 17, 21, 22]. Isolated fertilization membranes have been studied with the electron microscope [11, 25]. Attempts to estimate the thickness of the fertilization membrane have been made also with the polarization microscope [46] as well as with the interference microscope [26].

MATERIAL AND METHODS

Sea urchins, *Echinus esculentus*, *Psammechinus miliaris*, *Strongylocentrotus droebachiensis*, *Spatangus purpureus* and *Echinocardium cordatum*, collected on the Swedish west coast were used. After fixation in 1 per cent osmium tetroxide dissolved in sea water [21] the eggs and oocytes were dehydrated and embedded in butyl- and methyl-methacrylate according to Newman, Borysko and Swerdlow [33]. Other fixation methods have been tried and are described in a later section. The sections were made with the ultra-thin technique of Sjöstrand [49, 50], using a microtome designed by him [51]. As a cutting edge highly polished Schick razor blades mounted in a curved position were used [49]. The sections were floated on a liquid surface of 20 per cent ethyl alcohol according to Gettner and Hillier [9]. The sections were transferred to specimen grids covered with a thin formvar film. They were examined in the electron microscope without dissolving the methacrylate. An RCA EMU 2c electron microscope was used with standard objective aperture and with three apertures in the projector lens and reduced condensor aperture.

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[1] The publication of this and the following ten papers, presented in connection with the Symposium on the Physiology of Fertilization, Palermo, April 26–30, 1955, have been partly defrayed by funds from the International Union of Biological Sciences and from the Consiglio Nazionale delle Ricerche, the Sicilian Department of Health and Fishery.

RESULTS

Fine Structure of the Cortical Granules

Echinus esculentus.—The cortical granules are easily recognized in the mature *Echinus* egg. All of them are located in the outer layer of the egg, the cortex, which is about 1μ in thickness (Fig. 2). Their size is around 0.8μ (Table I). One granule consists of an outer double membrane of about 90 Å, usually four peripheral inclusions on the level of the section and some central lamellae. Each lamellae appears darker at its margins than in the middle parts and it has a mean thickness of 330 Å (Table I).

When found in the oocyte the cortical granules are scattered throughout the cytoplasm and may or may not have the same structure (Fig. 1). They may appear to consist of one or a few lamellae in a spiral or a concentric arrangement with three or four layers from the center to the periphery. In the examined stages the outermost layer is not complete but at some places desorganized in structureless matter, which does not take up osmium as readily as does the lamella. Where these marginal bodies are thicker than 0.1μ the inner lamellae are pressed together in a wheel-shape formation and the spiral order is lost. There are all gradations between a cortical granule of the "spiral type" and one of the "mature type" with peripheral inclusions.

Psammechinus miliaris.—The appearance of the cortical granules in the *Psammechinus* oocytes agrees well with that of the cortical granules in the *Echinus* oocytes. Thus one finds about three layers of an osmium stained lamella inside an outer membrane. The size and other dimensions correspond well to those of the *Echinus* oocyte (Tables I and II). The transformation of the granules, which parallels their transport to the cortex, is not as regular as that in the *Echinus* egg. A similar sort of disintegration of the peripheral part of the lamella seems to occur, but there is no resulting wheel-shape formation of the central parts (Fig. 9). The peripheral spaces formed in this way are so large that the total size of the cortical granule is increased in the mature egg. In the cortex of a mature *Psammechinus* egg there are also some considerably smaller cortical granules, mostly about 0.2μ. It is obvious that these are not laterally sectioned parts of larger granules since the outer membrane and lamella appear distinct. In these smaller granules the same structure appears as in the larger ones, although no disintegration of the lamella can be seen. There are not many intermediates between the small and the large granules.

Strongylocentrotus droebachiensis.—In the species *Strongylocentrotus droebachiensis* the cortical granules are of the same general structure as in the two species above, yet with the constituting lamella somewhat thinner. The cortical granules are large and there are many layers of lamellae; as many as 13 layers may be counted in one granule from the center to the periphery, as in Fig. 3. The lamella appears evenly dark or it seems to consist of small vesicles (diameter about 100 Å). As in *Psammechinus* eggs beside the normal cortical granules there are small ones with one, two or a few layers; the size of the small granules ranges from 0.16μ. The different layers in a small granule may be connected to each other in a spiral order or appear free as concentric layers. One or a few lamellae rolled up in a spiral can give rise to these two pictures when cut in different planes. At regular intervals the lamellae seem to be connected to each other, the total structure thus somewhat resembling a *Nautilus* shell. In a large granule, especially one of the mature egg, the lamella is absent in a randomly located sector of the granule and a less osmiophilic diffuse network can be distinguished. There are about six cortical granules in an area of $10\mu^2$ of the cortex.

Spatangus purpureus.—In the eggs of the three regular sea urchins described above, the size of the immature cortical granules does not differ considerably from the mature ones, at least not in the examined stages. This does not seem to be the case in the eggs of the irregular sea urchin, *Spatangus purpureus*. —Oocytes of this species have not been examined, but in the mature egg the two different granule types exist, as well as transition types. The immature granules are found mostly in the subcortical region and have a total size of only 0.4μ (Table I). They consist of an outer double membrane and a tightly coiled lamella (Fig. 5). The latter has a thickness of about 90 Å and is stained with osmium at its margins.

In the cortex granules of another size, 0.9μ, are seen. These have quite a different aspect, the only morphological similarity between these and small granules of the "spiral type" is the thickness of the outer membrane. This is in both cases about 80 Å, which is a value between that of the outer membranes of the yolk granule and that of the mitochondrial outer double membrane. The appearance of the mature cortical granule is otherwise indistinct.

Table I

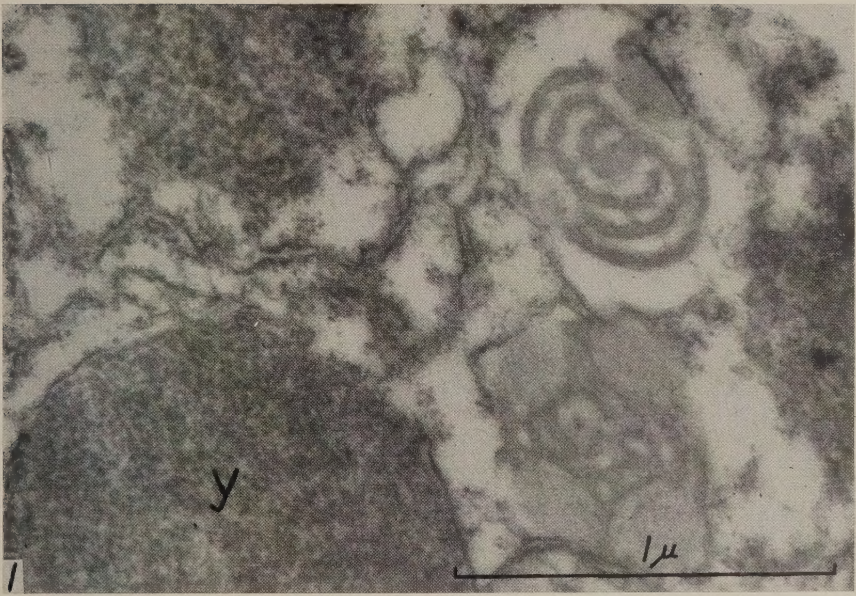
Dimensions of the sea urchin cortical granules. The values of the cortical granule diameters are given in microns, those of the lamellae and those of the spaces between two lamellae are given in Å units.

	Cortical granule diameter		Cortical granule lamella width		Distance between two lamellae	
	μ	σ	Å	σ	Å	σ
Echinus esculentus, oocyte	0.91 ± 0.06	0.17	322 ± 10	30	irregular	
Echinus esculentus, mature egg, osmium-fixed	0.79 ± 0.04	0.11	330 ± 14	44	,,	
Echinus esculentus, mature egg freeze-dry, osmium	0.89 ± 0.04	0.12	323 ± 14	45	,,	
Psammechinus mil., osmium-fixed	0.71 ± 0.05	0.16	364 ± 16	49	,,	
Psammechinus mil., uranyl-fixed	0.77 ± 0.06	0.19	334 ± 13	41	,,	
Psammechinus mil., HgClCH$_3$-fixed			396 ± 27	83	,,	
Psammechinus mil., formalin-fixed			341 ± 26*	64	,,	
Strongylocentrotus, osmium-fixed	0.92 ± 0.05	0.16	191 ± 3	10	188 ± 8	23
Spatangus purp., "immature granule", osmium-fixed	0.39 ± 0.02	0.05	90 ± 5	16	irregular	
Spatangus purp., "transitional granule", osmium-fixed			99 ± 7*	16	,,	
Spatangus purp., "mature granule", osmium-fixed	0.90 ± 0.03	0.11	—			
Echinocardium cordatum, osmium-fixed	0.50 ± 0.02	0.06	93 ± 3	10	119 ± 13	39

The values have been calculated from ten electron micrographs, each giving a mean value of a few measurements; those marked with an asterisk from only six electron micrographs.

Fig. 1. *Echinus esculentus*, oocyte. Two cortical granules in the endoplasm; the upper right in its immature state, the lower right with peripheral spaces. Fixation in 1 per cent osmium tetroxide in sea water. Y = yolk granule. Magnification 56,000 ×.

Fig. 2. *Echinus esculentus*, cortex of a mature egg. The lower right white space is probably a pigment vacuole. Fixation in 1 per cent osmium tetroxide in sea water. Magnification 45,000 ×.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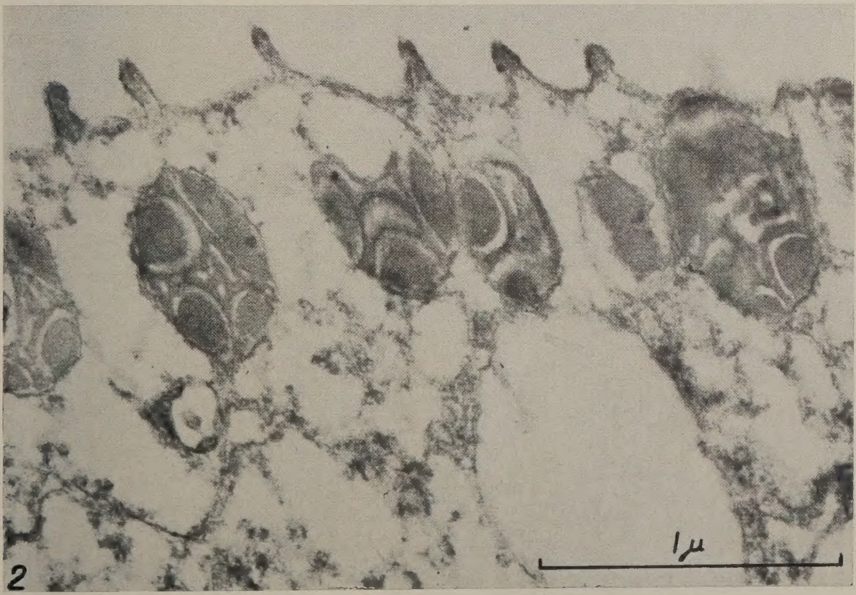
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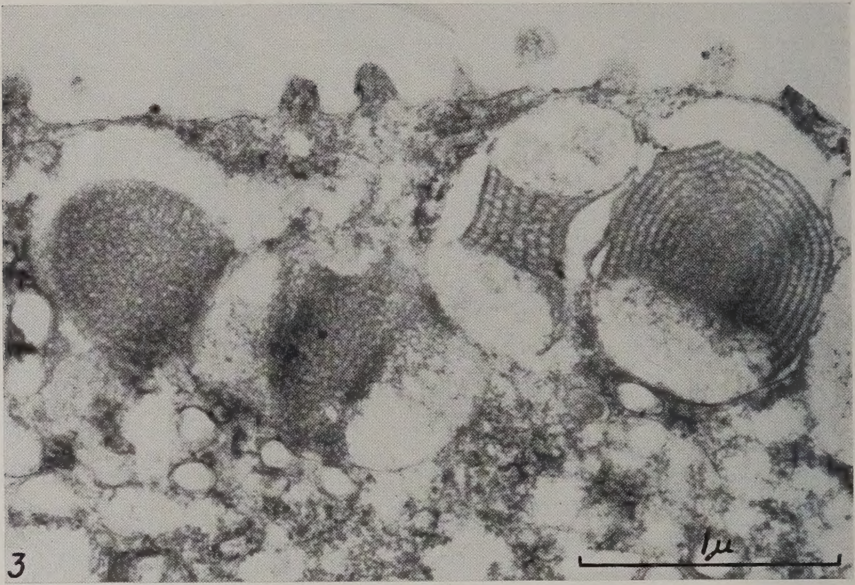
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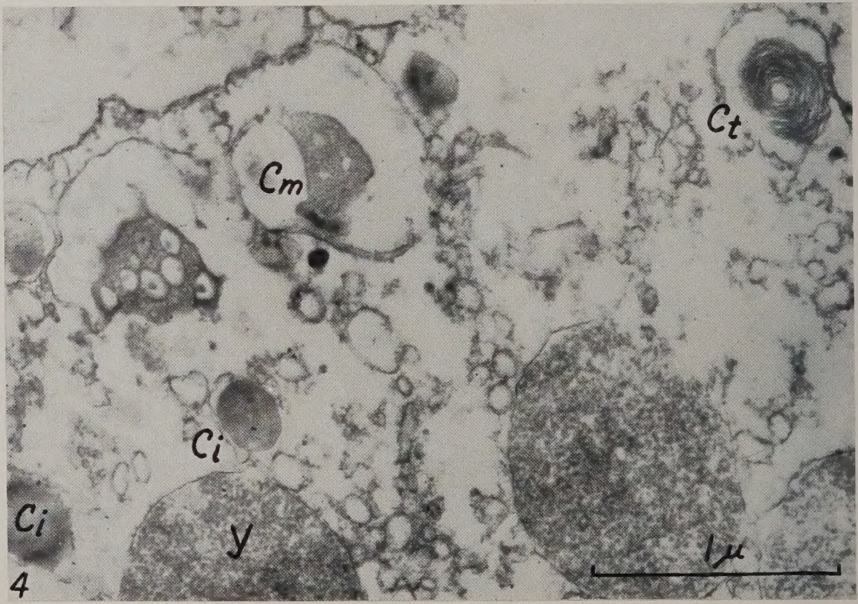
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Cm
Ct
Ci
Ci
y
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Table II

Dimensions of the cortex of the unfertilized and the fertilized egg and of the fertilization membrane. All values are given in Å units.

	Vitelline membrane	σ	Cortical granule membrane	σ	New cell membrane	σ	Fertilization membrane	σ
Echinus, esculentus unfertilized egg, osmium-fixed	98 ± 6	17	90 ± 5	14	—		—	
Echinus esculentus, unfertilized egg, freeze-dry, osmium			77 ± 4	10	—		—	
Echinus esculentus, fertilized egg, osmium-fixed	—		—		100 ± 5	15	630 ± 67*	164
Psammechinus mil., unfertilized egg, osmium-fixed	95 ± 5	14	74 ± 3	8	—		—	
Psammechinus mil., fertilized egg, osmium-fixed	—		—		79 ± 2	6	458 ± 32	100
Strongylocentrotus, unfertilized egg, osmium-fixed	89 ± 3	10	89 ± 4	12	—		—	
Strongylocentrotus, fertilized egg, osmium-fixed	—		—		84 ± 5	16	540 ± 22	69

The values have been calculated from ten electron micrographs, each giving a mean value of a few measurements; those marked with an asterisk from only six micrographs.
The fertilization membranes have been measured in eggs fixed
30 minutes after fertilization (*Echinus esculentus*),
5 minutes after fertilization (*Psammechinus miliaris*),
4 minutes after fertilization (*Strongylocentrotus droebachiensis*).

Fig. 3. *Strongylocentrotus droebachiensis*, cortex of a mature egg. Fixation in 1 per cent osmium tetroxide in sea water. Magnification 38,000 ×.

Fig. 4. *Spatangus purpureus*, cortex and endoplasm of a mature egg. The cortical granules can be seen in their three stages: *Ci*, immature granule; *Ct*, transitional granule; *Cm*, mature granule. Fixation in 1 per cent osmium tetroxide in sea water. Magnification 37,000 ×.

Fig. 5. *Spatangus purpureus*, cortical granule of the immature spiral type. Fixation in 1 per cent osmium tetroxide in sea water. Magnification 62,000 ×.

Fig. 6. *Echinocardium cordatum*, cortical granule. Fixation in 1 per cent osmium tetroxide in sea water. Magnification 65,000 ×.

Fig. 7. *Echinus esculentus*, survey picture of the outer layers of a mature egg fixed in methyl mercury chloride. Note the "electron stain" on the cortical granules (*C*) and the vitelline membrane (*V*). Magnification 8,000 ×.

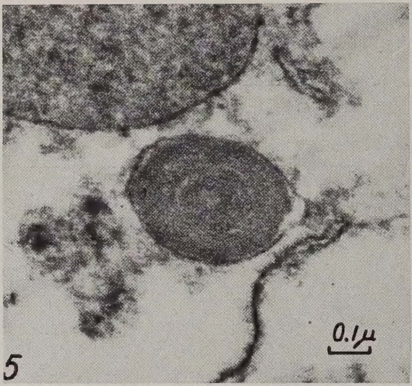
Fig. 8. *Echinus esculentus*, cortex of a mature egg fixed by the freeze-dry method followed by osmium tetroxide treatment. Magnification 32,000 ×.

Fig. 9. *Psammechinus miliaris*, cortex of a mature egg fixed in osmium tetroxide in sea water. Magnification 41,000 ×.

Fig. 10. *Psammechinus miliaris*, cortex of a mature egg fixed in uranyl nitrate. Magnification 41,000 ×.

Fig. 11. *Psammechinus miliaris*, cortex of a mature egg fixed in methyl mercury chloride. Magnification 41,000 ×.

Fig. 12. *Psammechinus miliaris*, cortex of a mature egg fixed in formaldehyde. Magnification 41,000 ×.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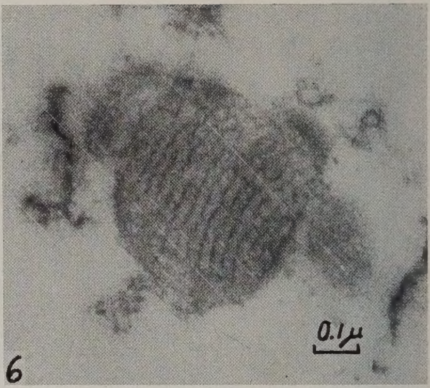
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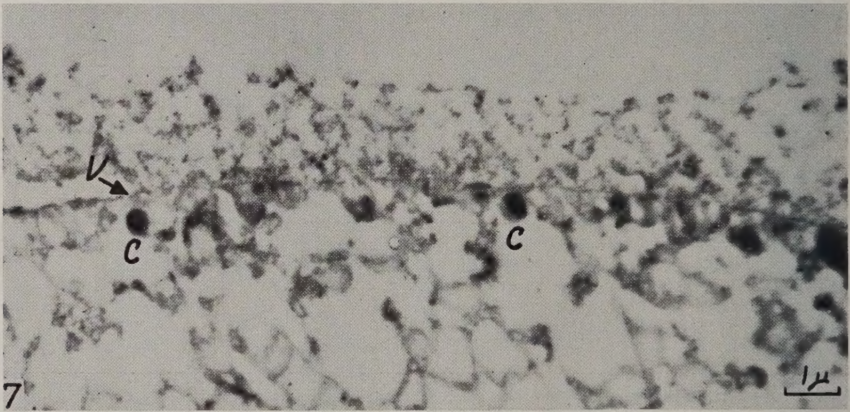
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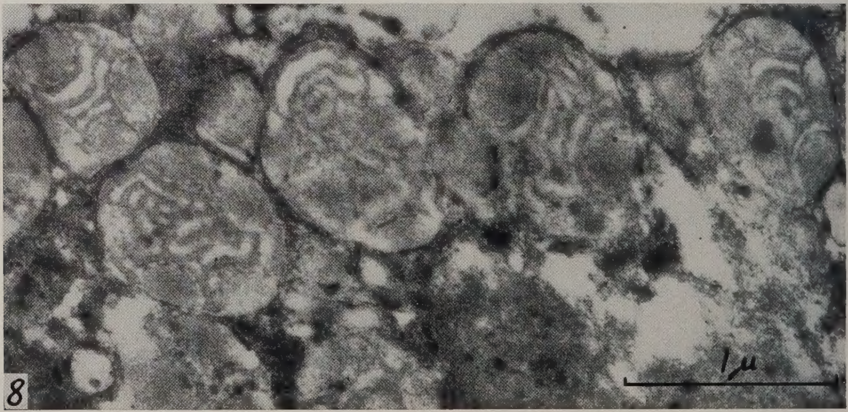
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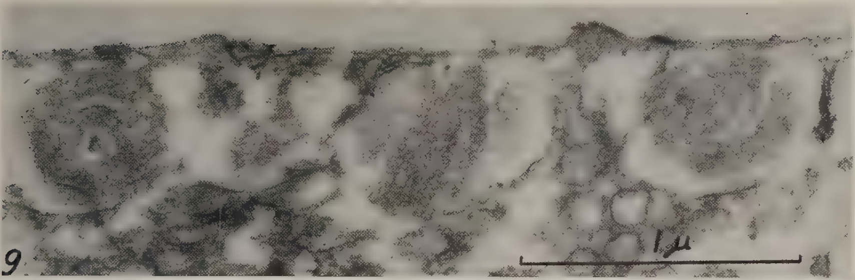
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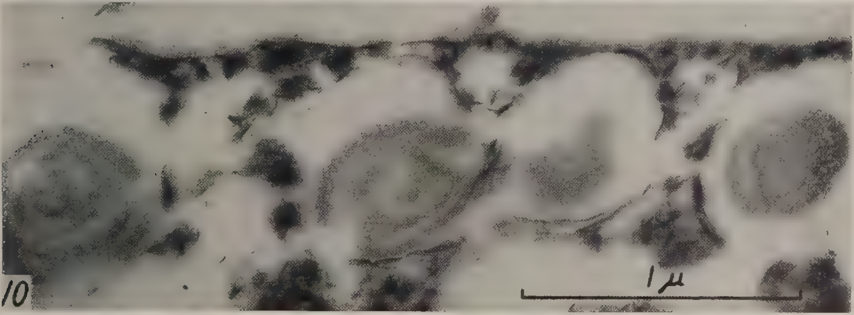
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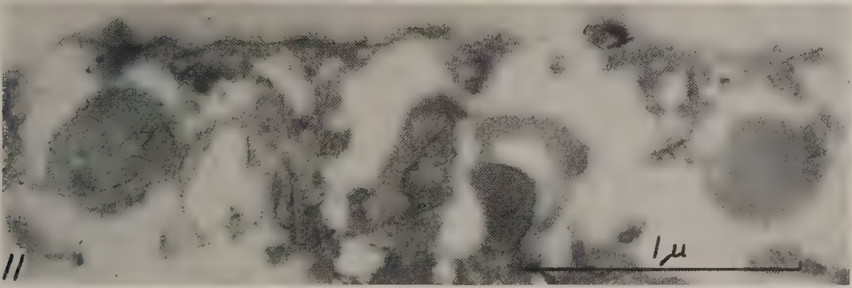
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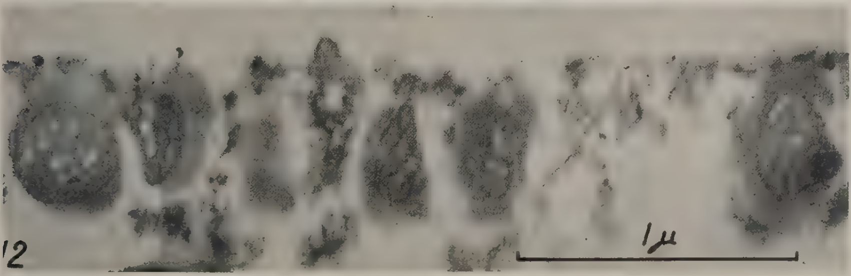
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There are, however, always many scattered, round cavities filled with non-osmium-impregnated substances. The presence of the transitional stages clearly demonstrate the connection between the small spiral granules and the large mature granules. Their size varies between that of the other two types and they possess a loose spiral structure. The number of coils of the lamella is the same in the small spiral granules as in the transitional stages, i.e. 5 to 7 layers from the periphery to the center. The larger diameter of the intermediates is due mainly to non-osmium-impregnated substances between the lamellar layers. In addition, the osmium-impregnated lamella has a slightly increasing thickness in the swelling intermediates. As a final step in the destruction of the spiral structure there is a vacuolization, starting at the center and at the periphery, and a disintegration of most of the lamella. In Fig. 4 granules in the three stages are shown.

Echinocardium cordatum.—Only mature eggs have been sectioned of the irregular sea urchin *Echinocardium cordatum*. The cortical granules of these eggs do not possess the common spiral order, but instead have a foliaceous structure, as seen in Fig. 6. This structure does not represent a length-sectioned spiral roll, as in that case the spiral structure should sometimes be observed. In a granule, with a diameter of 0.5μ, about 20 parallel lamellae are gathered with a spacing of 200 Å and a thickness of about 90 Å each.

The Influence of the Fixation Fluid

The necessity of having a control method beside the osmium tetroxide fixation of biological materials is a pressing but, on the whole, unsolved problem. Some attempts at attacking the fixation problem with regard to the cortical granules have been made and are presented here.

To test the influence of the ions in the fixation fluid the following variations have been examined: (1) Solutions made isotonic with sea water prepared with, (*a*) sodium chloride, (*b*) calcium chloride, (*c*) all main ions in sea water with exception of the calcium ion, (*d*) sucrose, (*e*) urea; (2) solutions of different tonicity, (*a*) sea water diluted to 30 and 60 per cent with

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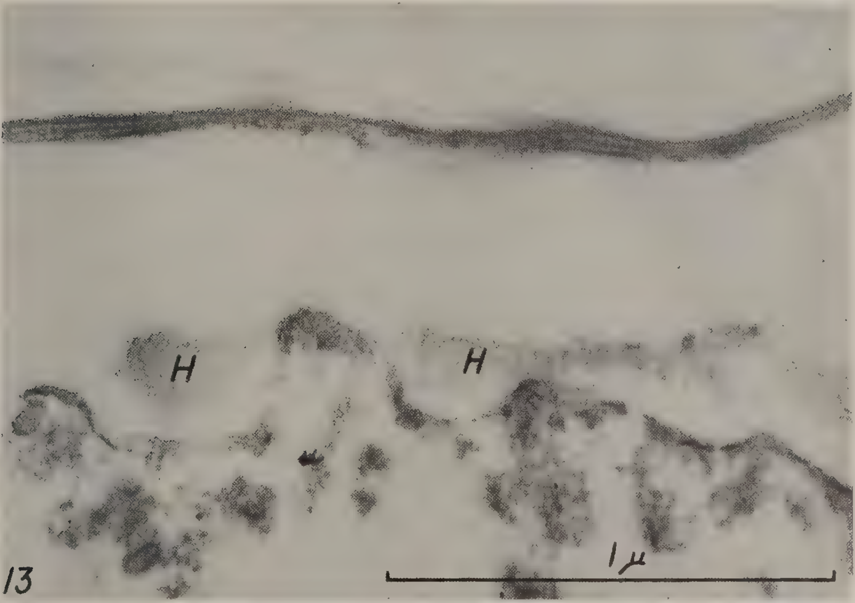
Fig. 13. *Psammechinus miliaris*, surface of an egg five minutes after fertilization. The elevated fertilization membrane shows a lamellar structure and a separated portion to the upper left side. In the perivitelline space near the egg surface the hyaline layer is seen. Fixation in 1 per cent osmium tetroxide in sea water. Magnification 66,000 ×.

Fig. 14. *Strongylocentrotus droebachiensis*, process of the fertilization membrane showing its dark outer rim. Magnification 95,000 ×.

Fig. 15. *Strongylocentrotus droebachiensis*, surface of an egg four minutes after fertilization. Under the fertilization membrane to the right side is one cortical globule (*Cg*). In the caves between the slender fringes of the new egg surface are two cortical granule remnants (*Cr*). Fixation in 1 per cent osmium tetroxide in sea water. Magnification 38,000 ×.



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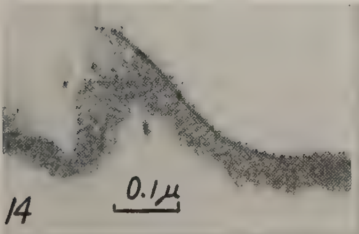

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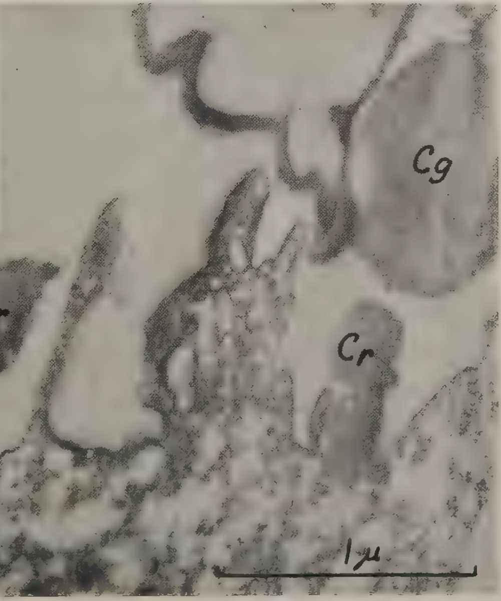

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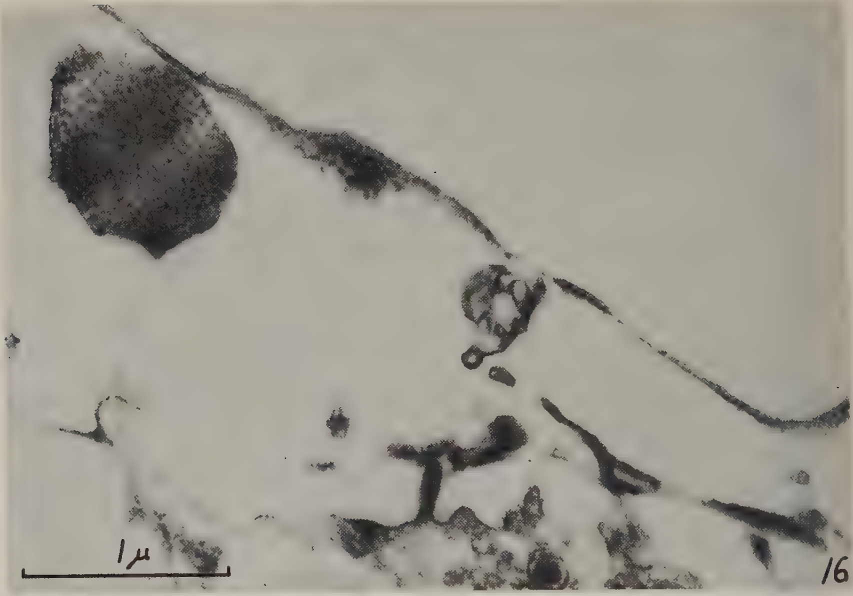
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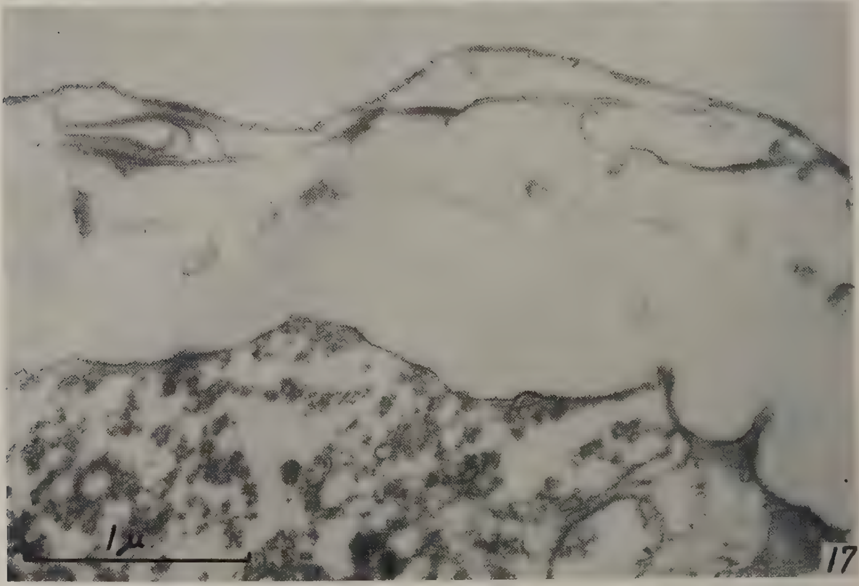
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distilled water, (*b*) sea water with sodium chloride added to a hypertonicity of 1.5 times the normal sea water tonicity. Furthermore, the pH value has been varied and kept constant at various values between 5 and 9 with acetate-veronal or phosphate buffers, and different temperatures of the fixation fluids have been tried. Fixation has also been performed in a drop of pure melted osmium tetroxide of 40° C.

In none of these cases were better results obtained than with 1 per cent osmium tetroxide dissolved in sea water and at a fixation temperature of 0° C. The buffer capacity of sea water is sufficient to keep the pH value between 7 and 8.5. It proved advantageous to perform the fixation, washing, dehydration and embedding during a time interval not exceeding eight hours. Likewise, it is best to have many steps in the dehydrating alcohol series: 50, 70, 90, 95 per cent and absolute alcohol. Fixation in near 100 per cent osmium tetroxide resulted in high contrast pictures of the egg, the structures of which seemed rather well preserved.

The ion-free fixation, solutions (1*d*) and (1*e*) above, resulted in inferior pictures, showing less orderly structures in the cortical granules, as well as in the mitochondria, and the ground cytoplasm was dissolved to a high degree. Otherwise, it was often difficult to correlate the ultrastructural picture obtained with the modifications of the fixation fluid. *The most important factor is the condition of the eggs chosen for fixation.* Eggs which have been at varying extent refractory to fertilization give unsatisfactory results, even if there is 100 per cent fertilization and development after treatment with sodium periodate [42]. Pictures obtained of such eggs show similarities to tissues subjected to postmortal changes as demonstrated by Rhodin [35] and Zetterqvist [54]. The necessity of fixing eggs with full fertilization percentage is therefore stressed. The best results are obtained when freshly collected animals are used, and when the time for the pre-treatment of the eggs has been reduced to a minimum. Removal of the jelly coat does not improve the fixation result.

It could be suggested that osmium tetroxide together with another fixing agent is necessary to get a more complete fixation of the cortical granules.

Fig. 16. *Echinus esculentus,* surface of an egg fertilized in the presence of 0.02 per cent lysozyme. A transformed cortical granule is seen to the upper left side, slightly to the right of this is the periphery of a cortical granule attached to the fertilization membrane and about in the center a cytoplasmic mass also attached to the fertilization membrane. Fixation in 1 per cent osmium tetroxide in sea water. Magnification 31,000 ×.

Fig. 17. *Psammechinus miliaris,* surface of an egg fertilized in the presence of 0.0005 per cent tannic acid. Cortical rods adhere to the fertilization membrane. Fixation in 1 per cent osmium tetroxide in sea water. Magnification 34,000 ×.

Evidence in this direction has been presented by McCulloch, who for this cell component in *Arbacia* eggs recommends a mixture of osmium tetroxide and chromic acid [21, 22]. This strongly acid solution has not been applied to eggs from any of the above mentioned species. On the other hand, fixation has been performed with a mixture of neutral 1 per cent potassium dichromate and 1 per cent osmium tetroxide. No difference has been detected between pictures obtained with this fixation and with the ordinary osmium tetroxide fixation.

Excluding osmium tetroxide a multitude of other substances can prove to be satisfactory in preserving one cell component or another. A substance may be of no value in light microscopy due to a slow penetration rate permitting only a very small volume to be fixed. This small fixed volume may be sufficient in electron microscopy. As the combination of fixation and "electron stain" is expected from salts of heavy metals, uranyl nitrate was tried. A 1 per cent solution of this salt was prepared and sodium chloride was added to sea water tonicity, the pH value was 3.6. The ground cytoplasm of *Psammechinus* eggs preserved in this solution appears as a conglomeration of small crystal needles. The cortical granules on the other hand have the same appearance as after osmium tetroxide fixation, except that the double contour of the outer membrane is lost (Fig. 10). The dimensions are otherwise identical (Table I). A fixation fluid similarly prepared but with 5 per cent formaldehyde added and a pH value of 4.5 gives the same results.

Of particular interest among fixatives is methyl mercury chloride, a heavy metal compound attacking the protein-bound sulfhydryl groups [2]. Using a saturated methyl mercury chloride solution (made isotonic with sodium chloride and buffered to neutrality with an ethanol amino phosphate buffer) the cortical granules stand out dark against the more diffuse cytoplasmic network (Fig. 7). In this figure the border of the egg surface appears as a dark line 150–200 Å thick, outside which is a jelly coat, about 3μ in thickness. The jelly coat is probably strongly contracted or partly dissolved as the thickness of this layer in the living cell is at least 20μ. The finer structure of cortical granules in *Psammechinus* eggs is identical after fixation with this agent and with osmium tetroxide, again with the exception that the outer double membrane is not preserved (Fig. 11). Furthermore the "peripheral spaces" in the granules react with methyl mercury chloride to give a rather dark "electron staining".

Another heavy metal compound known to react with the sulfhydryl groups is *p*-chloro-mercury-benzoate [4]. This substance was not sufficient in itself as a fixative for the sea urchin eggs. When *p*-chloro-mercury-benzoate was combined with neutralized formaldehyde fixation results were very similar to those obtained after a methyl mercury chloride fixation.

The chemistry of the reaction of cadmium chloride on biological material is also known to some extent. Baker [3] states that it forms additive compounds with lipids. This salt is used as a cytological technique together with formaldehyde in Aoyama's fluid. This fixing mixture (modified with sodium chloride to sea water tonicity and having a pH value of 6.8) was used on *Psammechinus* eggs and the results obtained are very similar to those obtained after the sulfhydryl group reagents or uranyl nitrate fixations with regard to the cortical granules.

A fixation mixture that does not preserve any fine details of the cortical granules is Bouin's fluid, i.e. glacial acetic acid, picric acid and formaldehyde.

It was of interest to see what effect formaldehyde alone would have. Fig. 12 shows a section of a *Psammechinus* egg cortex after formaldehyde fixation. A 5 per cent formaldehyde solution was used with sodium chloride added to isotonicity and magnesium carbonate to obtain a pH value of 7.6. It is remarkable that enough contrast can be obtained without introducing any heavy metal atoms. It is also notable that the cortical granule lamella appears darker at its margins, somewhat like an irregular double membrane, and that more diffuse substances can be discerned surrounding the coiled lamella. Consequently, the contents inside the outer membrane of the cortical granule in *Psammechinus* eggs are preserved in the same way with at least four simple fixatives: osmium tetroxide, uranyl nitrate, methyl mercury chloride and formaldehyde (Figs. 9–12 and Table I) and with as many fixative mixtures.

Of quite a different nature among the preservation methods is the freeze-dry method. This has been used in electron microscopy by Sjöstrand [47, 48, 52] and was also employed in this investigation. The eggs were fixed in a freeze-dry apparatus constructed by Sjöstrand and then treated in various ways. One group was directly embedded in the methacrylate, another group after three hours exposure to the vapour of an osmium tetroxide in a desiccator, and a third group was placed in a sea water solution of osmium tetroxide for four hours and then dehydrated and embedded. Good fixation is obtained with the latter method (Fig. 8). The cortical granule appearance and dimensions (Table I) agree with those of an ordinary osmium tetroxide fixation, perhaps with the exception that the lamellae are more split apart from each other resulting in a slightly larger granule. In the eggs that have been exposed to osmium tetroxide in a dry state this same structure is recognized in the cortical granules, although the pictures appear very diffuse. This is the case to still a higher degree with eggs that are directly embedded in a dry and "unstained" condition.

The Vitelline Membrane

Perhaps the best characterization of the vitelline membrane is that it forms part of the fertilization membrane at fertilization. The vitelline membrane is said to border the cortex and thus to be situated outside the cortical granules and below the jelly coat. It can be seen with the light microscope after treatment with distilled water [44]. Its counterpart in the present electron micrographs is vaguely seen as little space is left between the cortical granules and the jelly coat. A continuous thin line can be followed in the sections as an outer border of the cortex. It seems reasonable to compare this layer to the vitelline membrane, the existence of which has been inferred from light microscopic data. It has a thickness of only about 100 Å (Table II). It sometimes appears as a double membrane.

The cytoplasm in the cortex projects into processes (Figs. 2, 3, 4). In most of the *Psammechinus* eggs these are rather short and sparse, whereas in *Echinus* and especially in *Strongylocentrotus* eggs they are numerous and arranged in a regular hexogonal pattern. In these latter species they have a length of between 0.15 and 0.20μ and a width near the base of 0.15μ.

Changes Following Fertilization

The break-down of the cortical granules and the subsequent formation of the fertilization membrane from this component and the vitelline membrane is a rather rapid process. It is, therefore, not expected that all of the intermediate steps during this process can be obtained in an investigation such as this. Samples have been taken from eggs three to five minutes after addition of spermatozoa and fixed in osmium tetroxide solutions. In these eggs the fertilization membrane is elevated. The cortical granules are transformed and either found in the perivitelline space (between the egg surface and the fertilization membrane) or they have fused in the fertilization membrane.

A study of the new egg surface indicates that it is derived from the outer membrane of the cortical granule; it is a double membrane with a total thickness of about 90 Å (Table II). The new egg surface is fringed with depressions or caves representing the space occupied by the old cortical granules (Fig. 15). Often remains of the granule are left inside these caves. These remains have a lamellar structure although this is of a slightly finer dimension than that of the intact cortical granule. Although not so numerous, such cortical granule remnants exist even in eggs fixed some hours after fertilization, and they appear flattened in a tangential direction and situated just outside the fringes. Distinct lamellar remnants have otherwise disappeared after some hours of development; in most examined *Psammechinus* egg no distinct remnants can be found even in eggs fixed only ten minutes after fertilization. In these cases a structureless or fibrous matter fills the space between and immediately outside of the fringes (Fig. 13). This is the hyaline layer described in light microscope investigations [5, 32, 45]. The fringes of the egg surface perhaps lend support to this structureless matter. The fringes can be visualized with the light microscope by washing away the hyaline layer with Ca^{2+}-free sea water, when a radial halo-layer appears [14].

There is also another product of the cortical granules in the perivitelline space, the cortical globule [7, 37]. This is a spherical dense particle with a diameter of 1μ or more. In this particle the lamellar structure can no longer be seen, but in *Strongylocentrotus* it is a rather homogeneous mass apparently consisting of vesicles about 100 Å in diameter (Fig. 14). The size of the cortical globules and their sometimes bulging outlines may indicate that they are a product of several fused cortical granules.

Occasionally in the mature egg a few cortical granules remain one or a few microns under the cortex. In a fertilized egg these are also found and they keep there the same appearance and dimension. In the developing larva such granules have been found in the cortex of ectodermal cells of the gastrula.

The Fertilization Membrane

The thickness of the fertilization membrane has been measured to be around 500 Å, as is accounted in Table II. It is to be noted, however, that substances can be added to the membrane, largely at its inner side, to give this a far greater thickness. The fine structure of the fertilization membrane may vary. Often it is seen to be lamellar as the membrane of *Psammechinus* in Fig. 13 which is obtained after an osmium tetroxide fixation. This is also evident from cross-sections of eggs fixed in methyl mercury chloride or formaldehyde. It may be also rather homogeneous or finely granulated as in *Strongylocentrotus* eggs (Figs. 14 and 15). In this species there are regularly spaced processes on the outer side of the fertilization membrane. These have a height of 0.15 to 0.20μ and a width near the base of about 0.20μ, and are thus easily recognized as the "brush border" of the unfertilized egg. There are 6 per μ^2 in the fertilized state and 11 per μ^2 in the unfertilized state. These values are in good agreement with the estimation that the fertilization membrane has an area twice the egg surface. The processes are hollow and as well as the rest of the fertilization membrane they have a heavily stained outer rim (Fig. 14), whereas the main part of the material is finely granulated. This granulation resembles that of the lamella in the intact cortical granule in this species.

Treatment with Lysozyme

If the basic protein lysozyme is present during fertilization, the cortical granules are expelled but do not merge into the fertilization membrane in the normal way. A thin and granular membrane is then formed [10, 39, 41]. In this investigation experiments have been performed using 0.02 per cent crystalline lysozyme in sea water. No changes were revealed in the unfertilized egg. The fertilization membrane, on the other hand, is thinner than normally and with remnants of the granules (and also other cytoplasmic aggregates) adhering to its inner side (Fig. 16). The lamella is totally fractured and the granule appears as a mass of almost square-shaped particles, the size of which is slightly smaller than the width of the lamella of the intact granule.

Treatment with Tannic Acid

Light microscope investigations have shown that treatment with tannic acid results in a thicker fertilization membrane [8], as is the case with surface active agents especially in hypotonic sea water. It was of interest to inquire in what manner this thickening is accomplished. Fig. 17 shows a section of an egg fertilized in the presence of 0.0005 per cent tannic acid. It is not difficult to observe that the spiral lamellae have partly or totally unrolled. This is evident both from their thickness and finer structure. They could probably be compared to the cortical rods described by Runnström [37, 38]. At places where they adhere to the fertilization membrane the resulting complex membrane may be more than 1μ thick.

Treatment with Surface-active Agents

In one experiment *Psammechinus* eggs were treated immediately after sperm addition, with sodium lauryl sulfate resulting in a final concentration of this substance of about 0.0006 per cent and a sea water tonicity of 70 per cent of the normal. The fertilization membrane showed here still a new type of fine structure. Its outer portion is normal, but much cytoplasmic debris adheres to its inner border. Thus with this pretreatment a thick fertilization membrane is obtained. It is difficult in this case to present a mean value of the thickness, because of the irregularity of the granules and vesicle-like matter attached to the inner side. Another observation occasionally made in the same sections is that the hyaline layer may be continuous and laminated. The normally rather homogeneous matter consituting this layer can at time appear as two lamellae with a thickness of only about 150 Å.

DISCUSSION

In this paper the ultrastructure of the cortical granules, as well as some of their products, are described: the fertilization membrane, the cortical globules, the cortical rods, the hyaline layer and the new cell surface.

Cortical Granule Fine Structure

The first thing to be pointed out is that the cortical granules show characteristic and clear-cut species-specific differences, which could perhaps be shown to have some systematic value. It has long been shown that presumptive cortical granules appear in the interior of the oocyte in a stage earlier than the germinal vesicle breakdown. In light microscope investigations they are recognized by their staining reactions [27, 32], in electron microscope investigations by their complex texture [21, 22]. In the present study it is shown that these interior cortical granules have a structure different from that of the cortical granules in the cortex of the mature egg. Hence, it is possible to obtain a conception of the life span of the cortical granules of each species, and also to point out species-specificity in these events. In contrast to the great variations of the fine structure in the cortical granules in their mature state, their transformations during egg maturation follow parallel courses in the species examined. In all cases the first stages consisted of a rather regular structure, a lamella in a spiral or a concentric arrangement within an outer double membrane. In the mature granule in the cortex, on the other hand, the lamellar structure may be partly absent. More or less voluminous spaces appear, the majority of which are in the periphery. This distintegration of the lamella seems to be correlated with a decrease of the rigidity of the cortex during egg maturation [41, 53]. The smaller cortical granules, shown here to have the lamella remaining more or less intact, are reported by Allen [1] to be the last to leave the cortex during fertilization.

The species that has the most irregular appearance of the mature cortical granule is *Spatangus purpureus*, in which the lamellar structure is scarcely or not at all seen. In *Echinus esculentus* the disintegration of the lamella is regular compared to the other species. This results in a wheel-like picture with lamellar hub and spokes and peripheral inclusions, rather similar to the cortical granules of *Arbacia punctulata* described by McCulloch [21, 22] and Lansing *et al.* [17, 6]. Neither a lamellar nor a spiral structure was demonstrated in *Arbacia* eggs by these authors.

It has not been possible to trace the early origin of the cortical granules from any other cell component such as the mitochondria, the dictyosomes or the nucleolus. Neither the spiral structure nor the dimensions of their outer double membrane show any similarities to these cell components.

The Chemical Nature of the Cortical Granules

There are but a few histochemical staining experiments reporting the chemical composition of the cortical granules. Thus, Monné and Hårde [27] demonstrated protein and acid polysaccharides. A lipid compound was suggested by Runnström and Monné [43] and is also found to be probable in experiments by Ishikawa [16]. Hendee reported a negative phosphorus test in this component in eggs of the sea urchin *Lytechinus variegatus*, which excludes the possibility of appreciable amounts of nucleic acids or phospholipids [13]. The conception of the chemical nature of the cortical granules is also influenced in another direction by Hendee's investigations. As she reports quite different aspects of the cortical granules following fixation according to Fleming and Champy, the cortical granule has often been considered as a structureless particle of colloidal nature, which may be precipitated in different ways by different fixatives. The investigations performed by McCulloch on *Arbacia* eggs are to some degree on similar lines [21, 22]. He reports that the cortical granule has a recognizable fine structure with peripheral inclusions following fixation with osmium tetroxide and chromic acid, whereas the peripheral inclusions are not preserved in an osmium tetroxide or formaldehyde fixation. This is in contrast to the investigations by Lansing, Hillier and Rosenthal [17] and Cheney and Lansing [6], who demonstrated the peripheral inclusions of the cortical granules in the same species following fixation with a buffered osmium tetroxide solution. As an explanation different penetration rates of the fixatives could be proposed as well as occasional factors such as the quality of the eggs. In the present investigation extremely acid fixatives such as Champy and Fleming containing chromic acid together with osmium tetroxide have been avoided. A variety of different fixatives and fixative mixtures have been applied including the freeze-dry method of preservation. As practically all fixation methods used in this investigation give very similar pictures of the cortical granules the idea of a structureless colloid particle can be rejected. It may well be, however, that the disintegrated part of the cortical lamella is of colloid nature. This part is probably comparable to the peripheral inclusions in the cortical granules of *Arbacia* eggs. Studying the transformations of the cortical granules during the oogenesis one receives the impression that the same material may take the form of a lamella during early stages and of an amorphous and possibly colloidal substance in later stages.

Motomura demonstrated that the cortical granules could be washed away with a 1*M* urea solution within a few minutes [32] and Parpart and Laris state that the cortical granules explode in an isotonic non-electrolyte solution [34]. These facts provide an explanation for the lack of good fixation of the cortical granules with fluids containing non-electrolytes.

Through the present study it is indicated that the cortical granules are rich in sulfhydryl groups, the lamella as well as the peripheral spaces reacting readily with methyl mercury chloride. This is in good accordance with the theory suggested by Monroy and Runnström that disulfide linkages play a role during the tanning of the fertilization membrane [30].

The Vitelline Membrane

Considerable confusion prevails regarding both the morphology and the chemical nature of the vitelline membrane of the sea urchin egg. Most authors, however, agree on the existence of a formed layer in the unfertilized egg participating in the formation of the fertilization membrane. This layer has been demonstrated in a variety of ways. Chambers could visualize the vitelline membrane by microdissection of the egg of *Echinarachnius*, whereas the corresponding membrane in *Arbacia* eggs was too thin for demonstration with this method [5]. Runnström and co-workers have shown that the vitelline membrane separates from the underlying layers by hypotonic treatment of the egg [43, 44]. With trypsin digestion [23, 24, 38], or treatment with surface-active substances [38], or during aging of the egg [38] this characteristic is lost, and the egg cannot form a normal fertilization membrane.

In an electron microscope study by McCulloch a structureless membrane with an approximate thickness of 200 to 400 Å was shown, which was only conspicuous after an osmium tetroxide and chromic acid fixation [21]. This membrane is thought by McCulloch to correspond to part of the vitelline membrane and eventually to portions of the surface layers of the cortical cytoplasm. The cortical granules are reported to be in intimate contact with the cell surface. Hillier, Lansing and Rosenthal report that no vitelline membrane can be resolved in eggs of this same species, *Arbacia punctulata* [15]. In the present investigation a continuous limiting membrane is seen, separated by a narrow space from the cortical granules. This limiting membrane is strongly osmiophilic and has a thickness of approximately 100 Å (Table II). Evidence that this structure is the vitelline membrane, or part of it, arises from comparison with the fertilization membrane which has an outer rim of comparable thickness dissimilar to the rest of the membrane (Figs. 14 and 16).

Investigations of the chemical composition of the vitelline membrane have demonstrated its protein nature. This is concluded by Runnström and coworkers [38, 44] and Minganti [23, 24] from experiments with proteolytic enzymes. To some extent glycoproteins or else carbohydrates in other combinations with proteins could be included [39, 41, 42]. Layers of radially orientated lipid molecules in the surface layers of the sea urchin egg have been established by studies with the dark field microscope [19, 36], with the polarization microscope [21, 24, 45], from enzyme experiments [24, 45], as well as from staining experiments [24]. McCulloch also examined formalin or osmium tetroxide and chromic acid fixed eggs [21]; the loss of polarization in such eggs when immersed in media with a refraction index of 1.58 was taken as an indication that the surface layers under these conditions do not possess radially oriented lipid layers in detectable amounts nor tangentially oriented nucleoproteins. This can mean that the lipid moiety is removed during the fixation or that the orientation of the molecules causing polarization is lost, these may be lipids or proteins. To what degree the vitelline membrane contains lipids is difficult to state. The sensitivity of the polarization microscope is not sufficient to permit a complete analysis of the vitelline membrane isolated by hypotonic treatment [44]. With staining experiments Lansing and Rosenthal indicate the presence of ribonucleic acid in the vitelline membrane [18].

Although the present investigation does not deal with the chemical nature of the vitelline membrane, two observations in connection with this can be made. The part of the fertilization membrane that is derived from the vitelline membrane has a considerable rigidity, which is evident from the fact that the "brush border" of the *Strongylocentrotus* egg is maintained in its fertilization membrane. This is similar to the old observation that the fertilization membrane of the *Echinocardium cordatum* egg maintains the pear-shape of the unfertilized egg [36, 40]. Fixations with methyl mercury chloride as well as with the *p*-chloro-mercury-benzoate and formaldehyde indicate that the outer layer of the unfertilized egg is rich in protein-bound sulfhydryl groups. This may be related to the experiments of Lindberg [20], showing that the outer 200 to 500 Å layer of the egg surface is an active and metabolizing part of the egg.

The Fertilization Membrane

In a paper by E. N. Harvey, 1911, it is mentioned that the cortical granules disappear at the time of fertilization "apparently going to form the substance which passes out of the egg and hardens the fertilization membrane" [12]. The dual character of the fertilization membrane has been definitely established in works by Motomura [32] and by Runnström and co-workers [45, 46]. The course of events is followed in detail in later works by Runnström [37, 38] and these results have been confirmed in general by Allen [1] and Endo [7], among others. From polarization microscope studies Runnström concluded that the fertilization membrane is composed of lamellae with a thickness of less than the wave length of light. The total thickness of the fertilization membrane has been given different values according to the technique chosen. With the interference microscope a value of only 160 +80 Å is obtained by Mitchison and Swann [26], whereas estimations from polarization data have given far greater values [46]. When dried, metal shadowed and studied in the electron microscope, isolated and washed fertilization membranes of *Arbacia* eggs are reported by Harvey and Anderson to be 250 Å [11] and those of *Psammechinus* are reported by Mitchison to be 100 Å [25]. Mitchison calculated that the dried substance of the fertilization membrane is only 20 per cent and therefore the hydrated fertilization membrane has a thickness of 500 Å. More direct information about the thickness and the fine structure is obtained with sections through the fertilization membrane. Hillier, Lansing and Rosenthal state that the fertilization membrane of *Arbacia* eggs is around or less than 300 Å as measured from sections in the electron microscope [15]. As seen in Table II the mean value of the thickness is calculated for three sea urchin species. It should, however, be pointed out that these measurements are made on eggs no more than five minutes after the sperm addition (*Strongylocentrotus* and *Psammechinus*) and that slightly higher mean values may be obtained in later stages. The variation is great and values from adjacent parts of the fertilization membrane diverge to 50 per cent or more, beside the fact that adhering substances may increase the thickness manyfold as pointed out earlier. The necessity to examine fixed and embedded material results in an uncertainty, which has been reduced by fixing in different agents. Accordingly, the thickness has been measured on fertilization membranes from eggs fixed in formaldehyde or methyl mercury chloride; the same values have been obtained and the same fine structure appears.

The ultrastructure of the fertilization membrane in *Psammechinus* eggs is lamellar as predicted from polarization data [36], with a lamella thickness of about 100 Å. In *Strongylocentrotus* the lamellar structure has not been seen. This difference is easily compared to the appearance of the lamella of the cortical granules in these two species; that of *Strongylocentrotus* is evenly dark, whereas the lamella of *Psammechinus* has an appearance like an irregular double membrane with each osmiophilic compound being about 100 Å thick. In both cases the outermost part of the fertilization membrane deviates in "staining capacity" with osmium tetroxide (Figs. 13–16). This part is occasionally seen separated from underlying parts (Fig. 13), thus excluding the possibility that this rim results from a simple surface absorption of the osmium tetroxide. It is possibly this part of the fertilization membrane that maintains the shape of the egg surface and thus is the old vitelline membrane. Thus these results are in good agreement with the conception of the dual origin of the fertilization membrane.

The Hyaline Layer

Calculations of the cortical granule volume in the unfertilized and fertilized egg are of limited value, as little is known about the eventual changes of the granule volume after fertilization [41]. Such calculations have been made by Runnström *et al.* [46] and are here again performed with the species *Strongylocentrotus droebachiensis* and *Psammechinus miliaris*. In the first-mentioned species the egg diameter is 130μ (Swedish west coast), the cortical granule number 33,000 (0.6 per μ^2), and the mean value of each cortical granule within its outer membrane $0.38\mu^3$. This gives a total volume of all these granules in an egg of $12{,}500\mu^3$. This figure could be compared to the area of the fertilization membrane, including the surface of its processes, which is $125{,}000\mu^2$. With unchanged volume the cortical granule alone could thus give the fertilization membrane a thickness of 1000 Å. The corresponding value in the *Psammechinus* egg is calculated to be 1700 Å. The relatively small percentage of dwarf cortical granules is not included and does not influence this rough calculation.

These calculations could show that not all the material from the interior of the cortical granules goes to the fertilization membrane. Assuming no changes of the cortical granule volume during their expulsion, roughly half of the material must have other functions. However, the cortical granule could swell, as reported by Endo [7], and the proportions deviate much from this.

There is good evidence from electron micrographs that a substance from the cortical granules remains near the egg surface. This substance most probably is the hyaline layer. There are then two possibilities: all the granules may deliver part of their contents to the fertilization membrane and another part to the hyaline layer, or some may fuse to the membrane and others dissolve into the hyaline layer. Apparently during oogenesis a complete transformation from a lamella to a structureless mass is going on in *Spatangus* eggs and this mass again can be transformed to a lamella in the fertilization membrane. Therefore, it seems plausible that the entire content of each granule has the possibility of passing into the hyaline layer or into the fertilization membrane. In connection with this it is interesting to note that the hyaline layer after treatment with a surface-active agent may have a lamellar character, which is also evident from polarization data [46].

Similar staining results are reported in the cortical granules, the fertilization membrane and the hyaline layer by Monné and Slautterback [28] and to a great extent also by Motomura [32]. Hence it follows that the hyaline layer as well as the fertilization membrane could be a product of the cortical granules. Its appearance in time, as reported in light microscope investigations [32, 44, 46], fits in well with the time necessary for the cortical granule remnants to dissolve in a structureless mass, as seen in electron micrographs of eggs from different time intervals after fertilization.

The function of the hyaline layer is rather well known. During the cleavages of the egg the hyaline layer flows to the equator of the egg, and after the cleavage the blastomeres are pulled together so closely by this material, that their spherical shape is lost. With a number of substances the hyaline layer can be washed away and the blastomeres then become spherical and separated from each other. This can be done with Ca^{2+}-free media as shown by Herbst [14] and others [10, 32], with trypsin treatment [10, 45] and to some degree with some basic substances such as clupein [46] or lysozyme. The last substance has been used in many investigations to "precipitate" the cortical granules, resulting in a low and granular fertilization membrane. It has been shown here that lysozyme has no influence on the fine structure of the cortical granules in the unfertilized egg, in contrast to the basic substance caffein, as reported by Cheney and Lansing [6]. In the fertilization process, on the other hand, lysozyme has a marked effect, as described in an earlier section. The fracturing of the cortical lamella may explain the "precipitation" of the cortical granules, thus preventing the normal formation of the fertilization membrane and the hyaline layer.

The fact that cortical granules have been recognized in developing larva in the gastrula stage may perhaps also indicate that the cortical granules are not merely a material for the fertilization membrane formation. This may be associated with the observation made by Monné and Hårde that granules with the same staining properties as the cortical granules appear in the early blastula stage near the cell base at a time when the hyaline layer is thickening [27]. Hendee [13] and Motomura [32] observed that a few cortical granules remain in the surface layers of the fertilized egg.

The New Cell Surface

Because of their rapidity it is difficult to draw conclusions regarding the initial events following sperm attachment, in sectioned eggs fixed shortly after fertilization. Two of these phenomena, however, shall be discussed with respect to earlier light microscope analyses: the formation of the cell surface and of the cortical rods. According to Moser the cortical granules swell and form the perivitelline space and their walls fuse to form the new cell surface [31]. This mode of origin of the perivitelline space has been seriously criticized by Runnström and co-workers [37, 46] and others [7, 29], but the conception of the formation of the new cell surface from the cortical granule walls has not been distinctly rejected. The data given in the present paper make it further probable that the new cell surface of the fertilized egg is to a great extent the membrane of the cortical granules. The fringes of the fertilized egg have not the same appearance as the processes of the unfertilized egg but are often biconcave in the sections, as is the space between two cortical granules in the unfertilized egg. The thickness of the outer double membrane of the fertilized egg is about 90 Å (Table II). It is to be noted that in the fertilized egg most of the surface is oriented in a radial direction, due to these fringes, which is not the case in the unfertilized egg. These facts should be considered when the complex polarization data in eggs at fertilization is interpreted.

The break-down of each granule and its merging with the membrane is a rapid process, but the events may be delayed, for instance, by treatment with sperm lysin [37]. In this way Runnström could follow the sudden transformation of the isotropic cortical granules of *Psammechinus* eggs to birefringent rods with a length of 4–4.5μ. Such a tranformation of the granules into rods is sometimes seen in the untreated fertilized egg [7, 37] and it is thought to occur normally on the vitelline membrane under the action of some agents secreted from the egg. The rapidity of this transformation process can perhaps be explained as a mechanical process. By assuming that the cortical lamella, with a total length of some microns, is wound spirally within the outer membrane of the cortical granule, this unit can act as a spring. The picture obtained in this study of the cortical granule ultrastructure cannot explain why the lamellae subsequent to fertilization appear as narrow rods in light microscopy and it is not possible to state if one granule can give rise to several or just one rod. The total length of the cortical lamellae is between 2 and 3μ as measured in oocytes and mature eggs of *Psammechinus miliaris*. The cortical rods obtained after tannic acid treatment show the lamella unrolled with a length of between 1.5 and 3μ. The structure of the cortical rods is very much the same as that of the fertilization membrane.

SUMMARY

The ultrastructure of the cortical granules, of the vitelline membrane and of their products in sea urchin eggs has been studied by means of electron microscopy in ultra-thin sections prepared according to the technique of Sjöstrand.

1. The cortical granules exhibit clear-cut species-specific differences in the five sea urchin species examined. In four of the species the cortical granules have been followed from a rather regular "immature" state in the endoplasm, where the interior of the granule appears as lamellae in a spiral or concentrical arrangement. During egg maturation the cortical granules move to the cortex and the spiral order may be more or less lost, and peripheral spaces appear.

2. Beside the normal cortical granules there are also dwarf cortical granules of a submicroscopical size. These have the same ultrastructure as the larger ones except that no peripheral spaces appear.

3. To test the reliability of the osmium tetroxide fixation the following fixatives have been employed, all of which gave approximately the same results with regard to the cortical granule inner structure: uranyl nitrate, methyl mercury chloride, formaldehyde, Aoyama's fluid, osmium tetroxide + potassium dichromate, formaldehyde + *p*-chloro-mercury-benzoate and the freeze-dry method followed by osmium tetroxide treatment.

4. There is a continuous limiting membrane of the unfertilized egg with a thickness of about 100 Å. This is probably the vitelline membrane or part of it.

5. The outer layer of the cortex and the cortical granules are rich in sulfhydryl groups.

6. The fertilization membrane is shown to be composed of two different layers, the thin outer part is probably the vitelline membrane, the main

part of it is derived from the cortical granules. Thickness and fine structure of the fertilization membrane are described.

7. There is strong evidence that the hyaline layer is formed from the cortical granules that do not merge with the fertilization membrane. The fine structure usually is amorphous or fibrous. Treatment with surface-active agents may give the hyaline layer a lamellar character.

8. Cortical granules have also been found in embryos and these could be supposed to form cell cementing substances.

9. Most of the new cell surface of the fertilized egg is derived from the cortical granule outer membrane.

10. The granular fertilization membrane obtained after a lysozyme treatment is described.

11. Cortical rods, here obtained with tannic acid treatment, are shown to have the same fine structure as the main part of the fertilization membrane and are probably the cortical granule lamellae unrolled.

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MECHANISM OF FERTILIZATION BY RATE DETERMINATIONS[1]

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During the last century, an increasing number of chemical reactions in biologic processes have been revealed, and at the same time methods used in physical chemistry for studying chemical processes have been used more and more often in the study of biological processes.

In chemistry, one important field is the investigation of the mechanisms of reactions under various experimental conditions. In biology the corresponding problem is very complicated. However, in the case of fertilization, a pioneer work in investigating the mechanism by which eggs and spermatozoa combine has been done by Rothschild and Swann [16] by measuring the fertilization rate [3, 14] at various sperm concentrations.

The collision hypothesis

It seems appropriate to review briefly those parts of Rothschild and Swann's investigations, which are of essential interest for the present paper. Rothschild and Swann [16] tested the hypothesis that the fertilization reaction could be treated as a chemical reaction of the first order. The probability for every egg in a fertilization mixture to be fertilized in unit time was presumed to be the same. It was further presumed that spermatozoa move randomly as gas molecules and just by chance collide with an egg, whereby the collision can either be successful, i.e. the egg is fertilized, or not successful, i.e. the egg is not affected at all.

If the collision hypothesis were true, every egg would on an average need the same number of collisions with spermatozoa to be fertilized, regardless of the concentration of the spermatozoa. The fertilization rate of *Psammechi-*

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nus miliaris was measured at various sperm concentrations, and the number of collisions, on an average necessary for fertilization, was calculated. The result showed that the number was not independent of the sperm concentration, but increased with increasing sperm concentrations. It was thus proved that fertilization cannot be treated mathematically as a chemical process of the first order. Rothschild and Swann [16] discuss several sources of error and make the comment that the deviation from the hypothesis "may be due to sperm-sperm interactions of a physical nature".

Thus the collision theory—which it seemed reasonable to test first because of its simplicity—proved to be invalid. One consequence of this is that we should try to find another hypothesis for the mechanism of action of spermatozoa in fertilization.

Another consequence is that there is no longer any real reason to calculate a reaction rate constant for fertilization in the same way as it is calculated for a first order chemical reaction. One essential difference between chemical reaction and fertilization should be noted here. In chemical reactions, all molecules of the same kind have the same probability of reacting in unit time, and the molecules which have not reacted after some time have the same properties as at the beginning. In the case of fertilization, however, it is rather unlikely [8, 9] that all eggs are exactly alike, i.e. have the same probability of being fertilized in unit time. On the contrary, we must assume that there is a normal biological statistical variation in quality between the eggs, just as well as there is a difference in quality between the spermatozoa, as has been shown by Rothschild [15]. It is rather unlikely that fertilizable eggs, which have after some time not yet been fertilized, have not changed their properties. This point of view is supported by the observation of Hultin and Hagström [8] that the supernatant from sperm has influence upon the fertilization rate. Thus the quality of the eggs in the fertilization mixture changes with time owing to the influence of one or several substances in the sperm medium and probably also because of direct contact with the spermatozoa. This implies that the eggs in the fertilization mixture do not retain their original quality as do molecules which have not reacted in an ordinary chemical reaction mixture.

These considerations make it reasonable to introduce a statistical method for describing the course of fertilization experiments and thereby to make use of probit analysis for calculating the 50 per cent fertilization time, FT_{50}, also called the half fertilization time, for a group of eggs in the same way as the 50 per cent lethal dose LD_{50} is calculated in toxicity determinations [2]. The calculation of FT_{50} is described in a paper by Hultin and Hagström [9].

The orbit hypothesis

In this article, the present author wishes to suggest another hypothesis than the collision hypothesis for the approach to the mechanism of fertilization. It is known [4, 7, 8] that spermatozoa give off substances which have influence upon fertilization. The direct contact between the spermatozoa and the egg surface may also be important. It seems reasonable to assume that the spermatozoa which are near the eggs (the spermatozoa may, of course, come and go by their own movements) have a considerably greater influence than those which are at some distance. This can also be expressed so that only those spermatozoa which form what can be called the first orbit round the eggs, have a prime influence. It is self-evident that in this orbit there is space only for a limited number of spermatozoa.

It is also reasonable to assume that, as the sperm concentration is increased in the fertilization mixture, the number of spaces occupied by spermatozoa in the first orbit is also increased. From the presumptions made we must expect the fertilization rate to be proportional to the number of spermatozoa around the eggs, and that—with increased sperm concentration in the fertilization mixture—the fertilization rate does not increase indefinitely, but approaches a limited value. The fertilization rate should thus by this theory be proportional to the number of spermatozoa in the first orbit.

The theory for the mechanism for fertilization will on these presumptions mathematically be very similar to Henri's [5] theory (which was taken up by Michaelis and Menten, [13]; cf. [6]) for the influence of substrate concentration upon enzyme reaction rates, and to the adsorption theory by Langmuir [10].

Equations for the influence of sperm concentration on the rate of fertilization can thus be deduced in the following way.

Let FT_{50} be the period after which 50 per cent of the eggs have been fertilized when the number of spermatozoa in one ml of the fertilization mixture is n, lim FT_{50} the limit value of this period for increased spermatozoa concentration (polyspermy is left out of consideration here), N_f the number of free places in the first orbit round an egg, N_o the number of places occupied by spermatozoa, and $n_{\frac{1}{2}}$ a sperm constant (which will turn out to be the number of spermatozoa in 1 ml if the fertilization rate $1/FT_{50}$ is half the limit fertilization rate $1/(\lim FT_{50})$, i.e. the half rate concentration of the sperm).

If the rate by which spermatozoa come to the orbit, is proportional to the sperm concentration n and to the number of free places N_f, and if the rate by which spermatozoa leave the orbit is proportional to the number of occupied places N_o, we get

$$\frac{n \cdot N_f}{N_o} = n_{\frac{1}{2}}, \tag{1}$$

and, as the fertilization rate is presumed to be proportional to the number of spermatozoa in the first orbit round the egg,

$$\frac{1/(\lim FT_{50})}{1/FT_{50}} = \frac{N_o + N_f}{N_o}, \tag{2}$$

$$\frac{FT_{50}}{\lim FT_{50}} = 1 + \frac{N_f}{N_o}. \tag{3}$$

According to eq. (1), $N_f/N_o = n_{\frac{1}{2}}/n$, and hence

$$\frac{FT_{50}}{\lim FT_{50}} = 1 + \frac{n_{\frac{1}{2}}}{n}. \tag{4}$$

Equations are suitable for graphical calculations if the plots of the experimental values lie about straight lines. Equation (4) can be given in three suitable forms, which correspond to the three suitable forms [1, 11] of the enzyme equation for the influence of the substrate concentration on the reaction rate, and which also correspond to the three suitable forms [10, 12, 17] of the adsorption equation for the influence of the concentration of the substance, which can be adsorbed, on the amount of adsorbed substance. We get the following expressions:

$$FT_{50} = \lim FT_{50} + n_{\frac{1}{2}} \cdot (\lim FT_{50}) \cdot \frac{1}{n}, \tag{5}$$

$$n \cdot FT_{50} = n_{\frac{1}{2}} \cdot (\lim FT_{50}) + (\lim FT_{50}) \cdot n, \tag{6}$$

$$\frac{1}{FT_{50}} = \frac{1}{\lim FT_{50}} - n_{\frac{1}{2}} \cdot \frac{1}{n \cdot FT_{50}}. \tag{7}$$

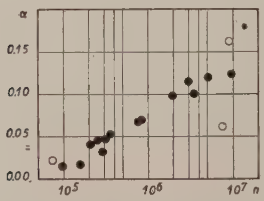
EXPERIMENTAL

The use of eqs (5), (6), and (7) for the calculation of the limit value of the 50 per cent fertilization time and the half rate concentration of the sperm will be demonstrated with Rothschild and Swann's [16] experimental values from their investigation of the fertilization rate of *Psammechinus miliaris*. They measured the fertilization rate constant α (calculated as for a chemical reaction) at various sperm concentrations, and from these measurements and from measurements of the mean speed of a spermatozoon and the egg radius they calculated the probability p of a successful collision. This probability at the various sperm concentrations in their experiments

TABLE I

Fertilization rate in *Psammechinus miliaris.* Rothschild and Swann's [16] measurements of the sperm concentration n and of the probability p of a successful sperm-egg collision; further, the recalculated value of the fertilization rate constant α, and, as calculated from α, the 50 per cent fertilization time FT_{50}.

n ml^{-1}	p	α sec.$^{-1}$	FT_{50} sec.
7.44×10^4	0.226	0.0225	30.8
9.84×10^4	0.121	0.0150	46.3
1.59×10^5	0.086	0.0168	41.3
2.11×10^5	0.152	0.0411	16.9
2.53×10^5	0.142	0.0456	15.2
2.89×10^5	0.099	0.0324	21.4
3.09×10^5	0.120	0.0466	14.9
3.67×10^5	0.115	0.0527	13.1
7.50×10^5	0.073	0.0671	10.3
8.29×10^5	0.068	0.0689	10.0
1.97×10^6	0.041	0.0977	7.1
2.99×10^6	0.032	0.1144	6.1
3.43×10^6	0.024	0.0998	7.0
5.11×10^6	0.020	0.120	5.8
7.50×10^6	0.007	0.062	11.2
9.18×10^6	0.015	0.162	4.3
9.62×10^6	0.011	0.124	5.6



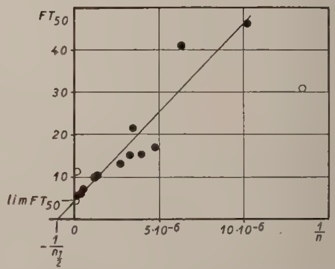
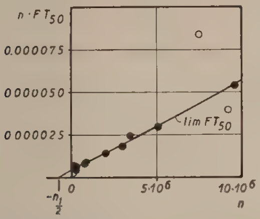


Fig. 1 (left). The reaction rate constant α according to Rothschild and Swann [16] plotted *versus* the logarithm of the sperm concentration n. Fig. 2 (right). Graph for the calculation of the limit 50 per cent fertilization time lim FT_{50} (the intersection between the fitted line and the axis of ordinates) and the inversed value of the half rate concentration, $1/n_{\frac{1}{2}}$ (the negative value for the intersection between the fitted line and the axis of abscissas).

is published as their Table 2. From this table the rate constant α has been recalculated and the 50 per cent fertilization time FT_{50} computed. The values are given here in Table I and plotted in Figs. 1–4.

In Fig. 1 α is plotted versus log n. Plotting is often done in a similar way for enzyme and for adsorption computations. The plots lie about a sigmoid curve. In Figs. 2–4 the plotting of FT_{50} and n is done according to eqs (5), (6), and (7), respectively, in order to make it possible to fit a straight line to the plots in each figure and to read from the line the limit 50 per cent fertilization time lim FT_{50} and the half rate sperm concentration $n_{\frac{1}{2}}$. Three of the experimental values differ considerably from the series. They are plotted as rings and have not been considered in fitting the straight lines.



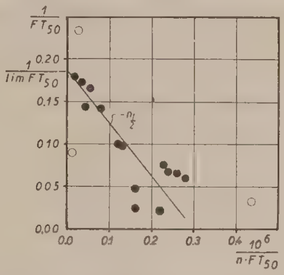


Fig. 3 (left). Graph for the calculation of the limit 50 per cent fertilization time lim FT_{50} (the inclination of the fitted line) and the half rate concentration $n_{\frac{1}{2}}$ (the negative value for the intersection between the fitted line and the axis of abscissas). Fig. 4 (right). Graph for the calculation of the limit fertilization rate 1/(lim FT_{50}) (the intersection between the fitted line and the axis of ordinates) and the half rate concentration $n_{\frac{1}{2}}$ (the negative value for inclination of the fitted line).

Plotting according to eq. (7) is most suitable for graphical calculations (Fig. 4), as the results can easily be read, but eq. (6)—which is used for Fig. 3—is most suitable for numerical calculations [6], as the abscissa values are independent and the accuracy of the ordinata values is—approximately—independent of the abscissa values. We get the following results from the calculations: lim $FT_{50} = 5.2$ sec., and $n_{\frac{1}{2}} = 0.7 \cdot 10^6$ spermatozoa per ml.

DISCUSSION

As already mentioned, Rothschild and Swann [16], whose experiments are used here, discuss several sources of error. Still another source of error, which was not known at the time when they published their paper, will be mentioned: the concentration of the fertilization rate regulators present in the sperm [8] was varied in the same way as the spermatozoa concentration. Thus the values of the 50 per cent fertilization time FT_{50} and the half rate sperm concentration $n_{\frac{1}{2}}$, which are read in Figs. 2–4, are influenced by the experimental variation in the concentration of the sperm medium. In future similar investigations it will probably be necessary either to use spermatozoa, whose original medium has been replaced by an inert medium, or to make up the various dilutions of the concentrated sperm suspension with sperm supernatant and take the same volume of these dilutions respectively for the various experiments.

In spite of these sources of error, the experimental values fit well—with a few exceptions—to the equations suggested on the basis of the orbit hypothesis. It is thereby of great interest to note that it was possible to use the same experiments for testing the orbit hypothesis as those used for testing the collision hypotheses; this was possible because of the detailed publication of Rothschild and Swann's experiments.

However, even if the experimental values fit a straight line in Figs. 2–4, the plots are rather scattered about the lines, particularly at low sperm concentrations. It is obvious that it would be of great interest to get more series of accurate measurements of fertilization rates at various sperm concentrations.

It would be very valuable, if, also in future investigations on fertilization rate, the number of unfertilized and fertilized eggs actually counted would be given, as this should make it possible to calculate statistically the accuracy by which hypotheses, which may be suggested later on, fit the experimental results.

The present author wishes finally to emphasize that the theory given here for the fertilization mechanism involves many simplifications, and that it will be necessary to develop it further, regarding, among other things, e.g. the time and the mechanism for the approach of the spermatozoa to the eggs.

SUMMARY

A theoretical interpretation is given for the influence of sperm concentration on fertilization rate: only those spermatozoa which are near the eggs have a prime influence, either chemical—as spermatozoa give off substances which have an influence upon the fertilization—or mechanical. As there is space only for a limited number of spermatozoa in the first orbit round the egg, the fertilization rate cannot incresae over a certain limit as the sperm concentration is increased. The influence of the sperm concentration n on the 50 per cent fertilization time FT_{50} is, according to this orbit theory, given by the following equivalent equations, which are suitable for the graphical computation of lim FT_{50} = the limit 50 per cent fertilization time, and of $n_{\frac{1}{2}}$ = the sperm concentration, at which the fertilization rate is half the limit

rate at high sperm concentrations; it is possible using these equations to fit straight lines to the plottings: $FT_{50} = \lim FT_{50} + n_{\frac{1}{2}} \cdot (\lim FT_{50}) \cdot 1/n$; $n \cdot FT_{50} = n_{\frac{1}{2}} \cdot (\lim FT_{50}) + (\lim FT_{50}) \cdot n$, and $1/FT_{50} = 1/(\lim FT_{50}) - n_{\frac{1}{2}} \cdot 1/(n \cdot FT_{50})$. The measurements of Rothschild and Swann on *Psammechinus miliaris* fit the equations well. The influence of fertilization rate regulators in the sperm medium should be eliminated in experiments for the determination of $\lim FT_{50}$ and $n_{\frac{1}{2}}$.

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THE VARIABILITY IN THE FERTILIZATION RATE[1]

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A THOROUGH investigation of a chemical reaction includes not only studying the participating molecules, but also elucidating the reaction mechanism, a problem which can usually be approached as a result of our efforts to interpret reaction rate experiments. In the same way, we must assume that in one of the most fascinating biological reactions, fertilization, a study of the kinetics whereby the number of unfertilized and fertilized eggs is determined at various times under various experimental conditions will give us information about the reaction mechanism. This point of view is supported by the fact that the chemical nature of the fertilization process has gained rapidly increasing interest.

Fertilization rate experiments were first carried out by Rothschild and Swann [6, 7]. It may be mentioned here that one aim of their rate experiments was to find out, whether or not the reaction mechanism between sperm and egg was the same as for a chemical reaction of the first order, i.e. whether the movements of the spermatozoa and the collisions of the spermatozoa with the eggs occurred as if the spermatozoa were the molecules of a gas. In such a case the average number of collisions necessary for fertilization should be independent of the spermatozoa concentration. It was shown by Rothschild and Swann's experiments, as pointed out by them, that the sperm behaved in another way, and one of us [4] has suggested another reaction mechanism for the mechanical behavior of sperm and eggs.

Rothschild and Swann's initial work on the rate of fertilization has great interest, because it was actually shown that a reaction mechanism hypothesis concerning fertilization could be tested by counting the number of unfertilized and fertilized eggs at various times.

The hypothesis that spermatozoa may behave like gas molecules and that all of the eggs may need on an average the same number of collisions with sperms in order to get fertilized, independently of the sperm concentration—a hypothesis which was tested but proved to be incorrect—led naturally to adopting the same calculating procedure for the fertilization rate as is used for calculating the rate constant in a first order chemical reaction.

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[1] Presented before the Symposium on the Physiology of Fertilization, Palermo, April 26–30, 1955.

The procedure was as follows. Samples of eggs were taken at different times and the number of unfertilized eggs and the number of fertilized eggs were counted. The relative number of unfertilized eggs, i.e. the quotient of unfertilized eggs divided by the whole number of eggs, was calculated, and the logarithm of this number was plotted versus time in a diagram. A straight line was fitted to the points, and the slope of the line calculated. If normal logarithms are used, we get the rate constant, if the negative value of slope of the line is multiplied by ln 10, i.e. by 2.3026. We can also get the half time for fertilization by dividing ln 2, i.e. 0.6931 by the rate constant.

In the experiments reported by Rothschild and Swann, the fertilization at constant sperm concentration proceeded in such a way as if all eggs had the same probability of being fertilized in unit time. However, in experiments on *Psammechinus miliaris* carried out at the Swedish west coast the present authors found [5] that it was in most cases necessary to assume the presence of at least two kinds of eggs: one with high fertilization rate and another with low fertilization rate. In addition it was necessary to consider the time lag in fertilization. With these additions, the calculating procedure used in our paper [5] was the same as that suggested by Rothschild and Swann. However, we expressly stated that—although the method for the calculation was convenient—it would obviously be erroneous to assume that all "rapid" eggs were identical, and that all "slow" eggs were identical. Furthermore, it must be presumed that there is a statistic biologic variation between the eggs so that all eggs have their own probability of being fertilized in unit time. Of course, these probabilities are distributed around one or several averages.

Whereas Rothschild and Swann started by presuming the mechanism for a first order chemical reaction to be valid for fertilization and characterized the fertilization reaction by the rate constant, we prefer to describe the fertilization reaction by the 50 per cent fertilization time FT_{50}, also called the half fertilization time, and by the standard deviation. In this article the probit analysis method [1] is suggested for calculating the 50 per cent fertilization time. It is possible by this method to calculate not only the half fertilization time for one or several groups of eggs but also to calculate for each group the distribution about its half fertilization time. It may be pointed out that this method is only descriptive and gives the distribution of the properties of the eggs in a group, probably as influenced by the distribution of the properties of the sperm used for the fertilization. Thus, by this way of calculation no hypothesis is introduced about the mechanism by which an egg and a spermatozoon meet for fertilization. However, as already mentioned, one of us [4] has suggested a mechanism for the mechanical behavior of eggs and spermatozoa.

We can distinguish between four kinds of fertilization rate variability for the eggs: the occurrence of several groups of eggs with different averages in one single female, the distribution of the eggs in one group about their average, the difference between females collected at the same time and place,

Table I

The number of fertilized (*F*) and unfertilized (*U*) eggs in the experiments.

April 22, 1954.

Sec.	Female no. 1				Female no. 2				Female no. 3			
	a		b		a		b		a		b	
	F	*U*	*F*	*U*	*F*	*U*	*F*	*U*	*F*	*U*	*F*	*U*
5	0	500	5	350	3	220	3	210			1	290
10	23	271	23	336	52	211	68	254			6	282
20	153	189	153	175	234	93	245	85			81	286
30	257	114	260	103	248	51	259	55			140	231
40	274	62	318	67	272	37	244	29			169	141
60	270	25	307	30	255	14	289	10			197	96
80	248	7	285	17	254	9	307	6			205	90
100	348	9	219	5	238	11	245	8			241	64
120	270	2	303	6	214	8	236	6			259	38
∞	300	0	300	0	500	0	500	0			500	0

May 6, 1954.

Sec.	Female no. 1				Female no. 2				Female no. 3			
	a		b		a		b		a		b	
	F	*U*	*F*	*U*	*F*	*U*	*F*	*U*	*F*	*U*	*F*	*U*
5	6	276	1	250	0	250	0	250	3	290	2	320
10	23	217	18	205	3	276	3	280	5	270	7	227
20	78	186	83	225	29	267	27	282	9	258	9	276
30	123	187	126	197	149	238	130	205	27	262	27	266
40	188	194	127	161	170	155	180	153	46	258	59	338
60	176	113	208	165	215	59	223	49	70	172	98	221
80	280	143	235	133	263	19	282	14	99	171	87	159
100	256	121	236	106	287	9	247	9	241	251	206	232
120	228	72	224	79	269	7	276	7	254	254	168	161
∞	312	6	300	2	270	2	302	3	279	3	302	1

and the difference between females collected at different times and places. All these kinds of variability will be considered in this paper.

It must be emphasized here, that the fertilization rate depends not only on the quality of the eggs but also on the quality of the sperm. Thus, the

TABLE I continued

May 13, 1954.

Sec.	Female no. 1				Female no. 2				Female no. 3			
	a		b		a		b		a		b	
	F	*U*	*F*	*U*	*F*	*U*	*F*	*U*	*F*	*U*	*F*	*U*
5	48	227	51	261	31	269	26	270	2	260	1	250
10	136	114	141	119	240	98	278	116	17	285	18	258
20	199	55	242	71	270	4	236	3	84	229	94	278
30	222	22	236	22	288	4	252	3	158	246	155	257
40	246	10	235	9	280	1	330	2	180	144	160	120
60	246	7	229	6	300	1	330	2	260	116	221	93
80	242	4	295	7	330	0	330	0	205	77	213	79
100	220	3	217	4	300	0	300	0	264	78	229	64
120	253	2	238	2	300	0	300	0	272	56	261	50
∞	300	0	300	0	300	0	300	0	310	4	254	3

May 22, 1954.

Sec.	Female no. 1				Female no. 2				Female no. 3			
	a		b		a		b		a		b	
	F	*U*	*F*	*U*	*F*	*U*	*F*	*U*	*F*	*U*	*F*	*U*
5	25	221	17	230	0	300	0	300	10	233	11	244
10	164	89	155	95	5	291	3	280	71	177	99	209
20	248	25	232	29	51	249	63	285	253	17	260	13
30	268	15	260	17	99	174	106	178	252	3	249	4
40	251	9	247	13	257	89	262	91	255	2	261	3
60	267	7	251	8	237	72	242	68	249	3	253	3
80	256	3	247	6	283	22	281	24	256	3	257	2
100	260	1	253	6	248	17	253	18	252	3	261	1
120	252	3	248	4	256	6	252	5	254	1	259	1
∞	278	1	280	0	300	0	300	0	300	0	300	0

results from one female actually give combined qualities of the eggs and the sperm. In the experiments reported here, the same sperm was used for all experiments carried out on the same day. Thus we get the actual difference between the eggs from females taken at the same time. The difference between females collected at different times includes the difference between the sperm, as new sperm was taken every day experiments were made.

EXPERIMENTAL

The principles, methods and conclusions in this work are of general validity, although for convenience we have chosen to illustrate the procedure described here with fertilization rate experiments carried out on the sea urchin *Paracentrotus lividus.*

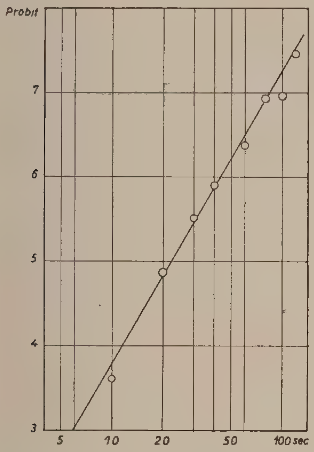
The fertilization procedure was a slight modification of the methods described earlier [3, 5]. On each of four days (April 22, May 6, May 13, and May 22, 1954) three females of apparently the best quality available were chosen for the experiments. The eggs of each female were removed and kept separately. They were washed twice by sedimentation and diluted to 200 ml with filtered sea water and divided into two equal parts. The same sperm was used for the series of 2×3 experiments carried out on one day. The sperm was stored in a test tube kept in ice water. Into two beakers 50 ml filtered sea water was poured; and sperm was added so that the final concentration in the fertilization mixture was 2×10^6 per ml. The two portions of eggs from one female were immediately added to the beakers. Samples were taken after 5, 10, 20, 40, 60, 80, 100 and 120 seconds and added to a solution of sodium lauryl sulphate, a substance which in 0.0007 per cent solution immediately stops fertilization without influencing the development of eggs which have already been fertilized. The times for taking samples were chosen with the aim of calculating the first order reaction constants [5, 6, 7]. It is evident from Fig. 3 that it would have been better to chose the times after the logarithmic scale, e.g. 5, 7, 10, 14, 20, 28, 40, 57, 80, 113, 160 sec. The procedure was immediately repeated with the eggs from the other females.

In all cases the fertilized eggs showed normal membrane elevation, normal cleavage and normal development. However, the rate of the development of the fertilized eggs was not the same in the various experimental series. The results from counting the eggs are given in Table I.

CALCULATIONS

If, in toxicity and infectibility experiments, the percentage of killed animals or infected organisms is plotted versus log dose, the points lie about a sigmoid curve [1]. This curve, obtained by integration of the normal statistical distribution curve, can be transformed to a straight line with probits. A probit table is included in Fisher and Yate's statistical tables ([2], Table IX).

A prerequisite for the probit analysis of the results from fertilization rate experiments is that all eggs can be fertilized. If only part of the eggs can be fertilized, the percentage of fertilized eggs in the fertilizable group should be calculated. The percentage of eggs which are fertilized after a long time can be taken as an estimate of that group. An example is given in Table II.



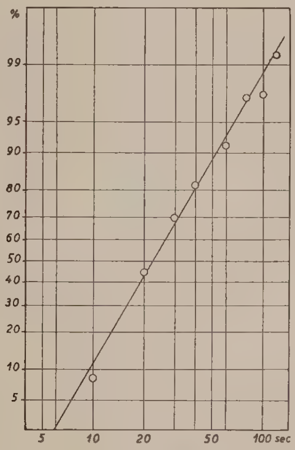


Fig. 1 (left). Female No. 1a of April 22. Probits corresponding to percentage of fertilized eggs in the fertilizable group plotted versus log time.

Fig. 2 (right). Female No. 1a of April 22. Percentage of fertilized eggs in the fertilizable group plotted versus time on probit-logarithmic paper.

The probit of the percentage of fertilized eggs in the fertilizable group is plotted versus log time—which is preferably done on semilogarithmic paper, as shown in Fig. 1. Then, if all the eggs are normally distributed about the half time, the points lie about a straight line. Probits correspond to abscissa values of the normal distribution curve, with the figure 5 added, and so, it is almost always possible to express probits as positive numbers. Hence, in the probit–log time diagram on semilog paper, the half time can be read at the probit value 5. It also follows from the definition of probits that the inverted value of the slope of the line is the standard deviation. If semilogarithmic paper is used, the standard deviation can also be calculated in either of the follwing ways. The time is read for the probit values 6 and 5 or for the probit values 5 an 4, and the higher number is divided by the lower. The logarithm of the deviation quotient thus obtained is the standard deviation. We can also read the time for probit values 6 and 4 and divide the higher number by the lower. The square root of this quotient is the deviation quotient, the logarithm of which is the standard deviation.

If probit-log-paper is available, the work is greatly simplified as neither probits nor logarithms need be looked up in a table (Fig. 2). Ordinary probit-paper is also very useful, especially if the times have been chosen properly.

A histogram of the results of the measurements, showing the log time distribution of the eggs is more informative than a diagram showing the accumulated percentage of fertilized eggs plotted versus log time. It can be made as follows (cf. Table II).

TABLE II

Computation of ordinata values for drawing the histogram. Female no. 3 of April 22.

Time sec.	Fertilized eggs, %	Log time	Difference in log time	Difference in % eggs	Ordinata
				0.3	
5	0.3	0.699			
			0.301	1.8	0.06
10	2.1	1.000			
			0.301	19.9	0.66
20	22.0	1.301			
			0.176	15.8	0.90
30	37.8	1.477			
			0.125	16.7	1.34
40	54.5	1.602			
			0.176	12.7	0.72
60	67.2	1.778			
			0.125	2.2	0.18
80	69.4	1.903			
			0.097	9.6	0.99
100	79.0	2.000			
			0.079	8.2	1.04
120	87.2	2.079			
			0.246*	12.8	1.04*
∞	100.0				

* For eggs which were fertilized before the first measurement or after the last measurement of the series, the corresponding surface is given as a triangle with the same height as the adjacent quadrangle.

The percentage increase of fertilized eggs is calculated for each time interval. The logarithm corresponding to the time interval is also calculated. If, for instance, we consider the time interval 40–60 sec, the logarithm corresponding to the interval is log $(60/40) = 1.778 - 1.602 = 0.176$. As the surface of a quadrangle in the histogram corresponds to the percentage of fertilized eggs, we get the height by dividing the difference in percentage for 60 and 40 sec, respectively, with the logarithm for the time interval.

If some eggs were fertilized before the first measurement or after the last measurement of the series, the corresponding surface is given as a triangle with the same height as the adjacent quadrangle. The histogram is preferably drawn on semilog paper (Fig. 3). If we prefer to use the relative number of fertilized eggs instead of the percentage, the percentage is divided by 100.

The normal distribution curve can be constructed in the following way.

We consider first that the surface of our histogram corresponds to the percentage of fertilizable eggs, which is 100 if all eggs are fertilizable, or to the relative number of fertilizable eggs, which is 1 if all eggs are fertilizable. The common tables of the normal distribution (e.g. [2], Table II) are calculated in such a way that the surface under the curve is 1. Hence, if we shall be able to use the same scale for the histogram and for the normal distribution curve, we must multiply the values for the ordinates of the normal distribution curve with the relative numbers of fertilizable eggs. We must also take into consideration that the normal distribution curve is tabulated with standard distribution 1. By the probit analysis we have found the standard deviation valid for the group of eggs under consideration. Even when the standard deviation is not unity, we wish still to have a surface under the curve, corresponding to the percentage of fertilized eggs. Hence, we must divide the values for the curve by the standard deviation. An example is given in Table III.

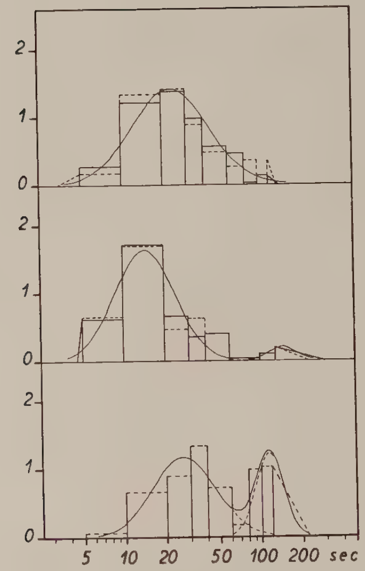
Table III

Computation of ordinata values for drawing the normal distribution curve. Female no. 3 of April 22. Standard deviation 0.232. 68.3 per cent of the eggs are in the group which is rapidly fertilized. The ordinata values of the normal distribution are multiplied by $68.3/(100 \cdot 0.232) = 2.94$.

Probit	Time seconds	Ordinates of the normal distribution	Ordinates for the fitted curve
4.6	21.4	0.3683	1.08
4.8	23.6	0.3910	1.15
5.0	26.2	0.3989	1.17
5.2	29.2	0.3910	1.15
5.4	32.6	0.3683	1.08

In the probit analysis diagram the times with deviation -0.4, -0.2, 0, 0.2, 0.4 etc. as measured in standard deviation scale, are read. That implies that the time is read for probit 4.6, 4.8, 5.0, 5.2, 5.4 etc. (It may be more convenient to move the straight line fitted in the probit analysis to the semilog paper where the curve is to be drawn.) On the semilog paper are now plotted the ordinates for the distribution curve, calculated as described, versus the corresponding time values (if semilog paper is not used, the ordinates are plotted versus log time), and the normal distribution curve is drawn through the points.

If it can be concluded from the histogram (Fig. 3) that the eggs belong to two different groups, the probit analysis can be made in the following way.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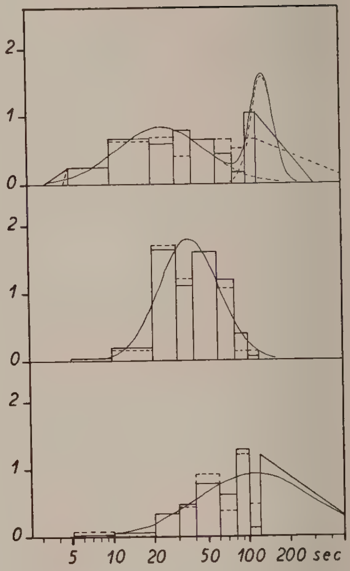
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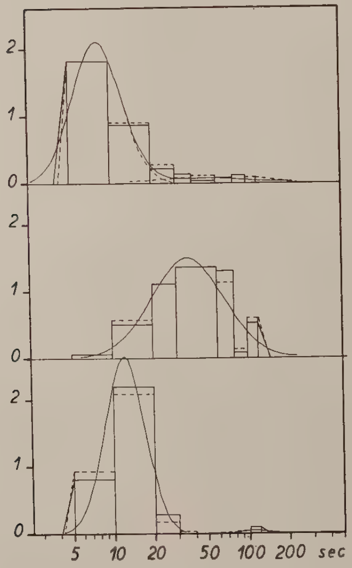
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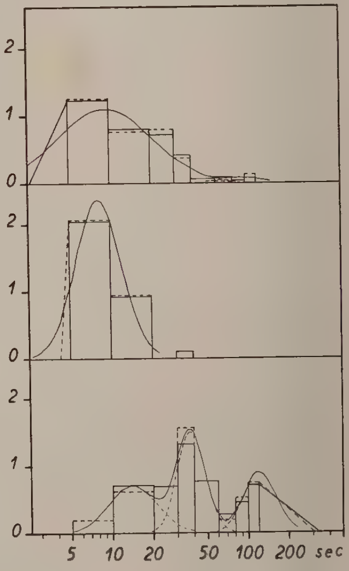
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sec

TABLE IV

Separation of two groups of eggs. Female no. 3 of April 22. The two groups are visible in the histogram Fig. 3 (cf. Table II).

Time sec.	Fertilized eggs %	Difference	Rapidly fertilized eggs %	Slowly fertilized eggs %	% of the group	Probit
		0.3				
5	0.3		0.3		0.4	2.34
		1.8				
10	2.1		2.1		3.1	3.13
		19.9				
20	22.0		22.0		32.2	4.54
		15.8				
30	37.8		37.8		55.3	5.13
		16.1				
40	54.5		54.5		79.8	5.83
		12.7				
60	67.2		67.2		98.4	7.14
		1.1	68.3			
		1.1				
80	69.4			1.1	3.5	3.19
		9.6				
100	79.0			10.7	33.8	4.58
		8.2				
120	87.2			18.9	59.6	5.24
		12.8				
∞	100.0			31.7		

The two groups are separated (Table IV) and the percentage of fertilized eggs in each group at the different times is calculated and the corresponding probits looked up in the probit table. The probits are plotted versus log time, preferably on semilog paper, and a straight line is fitted to the points. The half time is read at the probit value 5, and the standard deviation is also obtained as described in the example with only one group.

The procedure just described includes an approximation, as the overlapping parts

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Fig. 3. The distribution of eggs with regard to the time necessary for fertilization. Abscissa: log time. The ordinata scale is chosen so that the surface of a histogram and the surface between a curve and the abscissa gives the fraction of fertilizable eggs; i.e., the surface is 1 if all eggs are fertilizable. In the histograms, the full drawn lines and the lines of short dashes give the results from two measurements respectively. As to the curves, the full drawn lines give the most probable, computed distribution, and the lines of short dashes give the distributions of the various groups. The results of April 22, May 6, May 13 and May 22 are given at the top to the left, at the top to the right, at the bottom to the right and at the bottom to the left, respectively.

of the two normal distribution curves were truncated. This can be adjusted in a second approximation whereby the corrected experimental number of fertilized eggs at different times belonging to one group is obtained as the difference between the observed number and the theoretical number of eggs belonging to the other group as calculated from the line fitted in the probit analysis. However, if such a second approximation shall be of significant value, the number of samples in each experimental series must be considerably higher than in the experiments referred to here.

The results from the probit analysis are given in Table V and in Fig. 3.

Table V

The result of the probit analysis of the experimental values. % = per cent of the eggs in each group. FD_{50} = 50 per cent fertilization time in seconds for each group. SD = standard deviation for log time.

Date	Female no. 1			Female no. 2			Female no. 3		
	%	FT_{50}	SD	%	FT_{50}	SD	%	FT_{50}	SD
April 22	100.0	23	0.28	95.2	14.5	0.23	68.3	26	0.23
				4.8	140	0.10	31.7	113	0.10
May 6	67.6	24	0.32	99.1	38	0.22	99.3	110	0.43
	32.4	135	0.08	0.9	—		0.7	—	
May 13	96.8	9.5	0.35	100.0	8.2	0.17	32.3	14	0.18
	3.2	95	0.17				39.0	38	0.10
	or	or	or				27.5	125	0.12
	72.1	7	0.21				1.2	—	
	24.7	24	0.13						
	3.2	95	0.17						
May 22	92.1	8.5	0.18	100.0	37	0.27	98.8	12	0.15
	7.7	47	0.42				1.2	110	0.10

DISCUSSION OF THE CALCULATION

In most of the experiments, there is hardly any doubt as to the results of the probit analysis for the number of groups of eggs, although in a few experiments it would have been highly desirable to have samples from a shorter period than 5 seconds or from a longer period than 120 seconds in order to get a better estimate of average and of the standard deviation.

However, in some experiments the number of groups of eggs may be questionable.

In the samples taken at different times, only a certain limited number of eggs—about 250—were counted. Hence, in our samples, we can expect to get random statistical deviations from the most probable number of fertilized and unfertilized eggs. The question is whether or not the observed deviations from the hypothetical values are only random or if they are significant.

It is reasonable not to assume the presence of more groups than necessary. If, in a questionable case, we start by assuming the presence of only one group, we may find that the deviations are significant. We are then compelled to assume the presence of two groups. If, on the other hand, the deviations are insignificant, there may of course be two or even more groups, but we have no actual reason to assume the presence of more than one group. However, in such a case we get, of course, no support for denying the existence of several groups of eggs, if their existence is assumed because of other reasons.

As for the experiments reported here, the number of groups is questionable in three cases: No. 1 of May 13, No. 3 of May 13, and No. 2 of May 22 (Fig. 4). In the case of No. 1 of May 13, for example, the question is if we should assume the presence of only one group of eggs with half time 10 seconds and standard deviation 0.35—except the small group with half time about 95 seconds—or assume the presence of two groups of eggs, one with half time 7 seconds and standard deviation 0.21 and the other with half time 24 seconds and standard deviation 0.13. In Fig. 4, the full drawn normal distribution curve corresponds to the first assumption whereas the line of short dashes and the dotted lines correspond to the second assumption.

The significance of the deviations can be tested in the same way as in the case of dosage mortality tests the goodness of fit is tested by computing the χ^2 value; for χ^2 analysis the reader is referred to some text-book of statistics (e.g. [8]). The method is also described and exemplified in Fisher and Yate's statistical tables [2]. The calculations can also be done according to R. A. Fisher's maximum likelihood method which is exemplified in the paper by Rothschild and Swann [7] already referred to.

For female No. 1 of May 13 the probability that the deviation is random turned out to be as high as between 0.2 and 0.1 (only the experimental times up to 40 seconds were considered). The deviation is thus not greater than we can expect now and then in random sampling, and we have no statistical reason for putting forward a conjecture that there are two groups of eggs with half times 7 and 24 seconds, respectively, as indicated by the line of short dashes. However, we have no statistical reasons neither to deny the existence of the two groups.

In female No. 3 of May 13 (Fig. 4) we have another case where the number of groups of eggs is questionable. Except the group of eggs with half time of about 125 seconds, there is either one group with the half time 24 seconds and standard deviation 0.26, or two groups, one with half time 14 seconds and standard deviation 0.18, and another with half time 38 seconds and standard deviation 0.10. A χ^2 analysis shows that the probability that the observed deviations from the single curve are random is lower than the confidence limit 0.001. Hence the deviations are highly significant, and we have

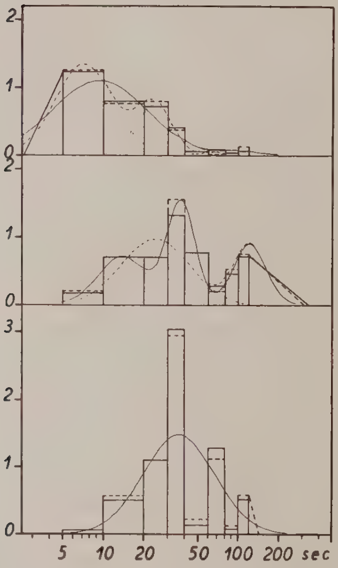

Fig. 4. Females, for which the number of statistical groups of eggs can be questioned. At the top: No. 1 of May 13. At the middle: No. 3 of May 13. At the bottom: No. 2 of May 22.

reasons to assume that there are two groups apart from the one with half time 125 seconds.

The third questionable case is female No. 2 of May 22 (Figs. 3 and 4; in Fig. 3 the results of the measurements at 40 seconds are omitted as they may be wild values). If the values at 40 seconds happen to be unrealiable, the curve of Fig. 3 gives an acceptable estimate of the distribution of the eggs; if the values at 40 seconds are significant, and it is not very likely that the results from two measurements which agree so well should be wild, there must be several groups of eggs. However, the shape of the histogram is so peculiar that a reliable interpretation can hardly be given without further experience.

It is evident from these examples that in fertilization rate experiments the actual number of unfertilized and fertilized eggs counted in each single experiment should always be given in order to make a statistical treatment of the results possible and also in order to make it possible in the future to use the results for testing new hypotheses.

CONCLUSION

This investigation shows that, even in selected material of apparently the best quality available, there are marked variations in the fertilizability and in the fertilization rate. In connection with the experiments it has been noticed that the ensuing development of the fertilized eggs is influenced by this variability.

It is remarkable that in several females there are obviously several groups of eggs with different average fertilization times, as if the groups of eggs had been produced at different times and were in different states of maturity.

It is evident from the results reported here that it is indispensable to check the state of eggs intended to be used for investigations in which material of a high uniformity is desirable.

SUMMARY

The rate of fertilization in a mixture of eggs and spermatozoa can be characterized by the time FT_{50} at which 50 per cent of the eggs are fertilized and by the standard deviation of the distribution about the 50 per cent fertilization time. The logarithms of the times, at which the individual eggs are fertilized, are—at least approximately—normally distributed about the logarithm of the 50 per cent fertilization time. The calculation of the 50 per cent fertilization time and of the standard deviation is easily made from a graph in which the probits of the percentage of fertilized eggs are plotted versus the logarithms of the times; cf. dosage–mortality curves from toxicity experiments.

There are four kinds of fertilization rate variability for the eggs of *Paracentrotus lividus*: the occurrence of several groups of eggs with different 50 per cent fertilization times in one single female, the distribution of the eggs in one group about their 50 per cent fertilization time, the difference between females collected at the same time and place and the difference between females collected at different times and places. The rate of the development of the fertilized eggs is different for different groups of eggs. The variability in fertilizability and in fertilization rate is remarkable even in selected material of apparently the best quality available. For the various groups of eggs

from 12 high quality females FT_{50} varied between 7 and 135 seconds. The standard deviation for the logarithm of the fertilization time for the eggs in the various groups differed between 0,1 and 0,4. The differences between the females collected at the same time and place were not noticeably less than the differences between females collected at different times and places. The variability is thus so great that it is indispensible to check the fertilization rate of eggs intended to be used for investigations in which material of a high uniformity is desirable.

The fertilization experiments were carried out by one of us (B. E. H.), whereas the planning and the evaluation of the results were done by the other (E. H.). We wish to express our thanks to Professor J. Runnström for his kind interest in our work. We are indebted to Professor R. Dohrn for the privilege of carrying out the experiments in Stazione Zoologica in Naples and to Dr. G. Bacci for providing the material. The investigation was financially supported by the Swedish Natural Science Research Council.

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CHANGES IN THE OPTICAL PROPERTIES OF THE EGG SURFACE AT FERTILISATION[1]

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Two of the optical properties of the sea urchin egg surface which change at fertilisation are the birefringence and the scattering power. These can be detected by special forms of microscopy, and they are especially valuable in giving information about the sub-microscopic structure of living cells without damaging them. They are, however, by no means easy to interpret.

A polarising microscope will detect the presence of a birefringent structure, and birefringence is usually associated with the presence of orientation. The orientation may be at a molecular level, when the resulting birefringence is called intrinsic birefringence, or at it may be the level of micelles or molecular aggregates when the resulting birefringence is called form birefringence. These two types of birefringence can be distinguished by immersing the structure in liquids of high refractive index e.g. potassium mercuric iodide or various organic liquids. Providing the liquids penetrate into the spaces between the micelles, the form birefringence will vanish when the refractive indices of the liquid and of the micelles are the same. Intrinsic birefringence of the other hand is unaffected by the refractive index of the immersion liquid. The separation of these two types of birefringence is a necessary part of any complete polarised light analysis, but it is very difficult, if not impossible, to do with living cells since they cannot be placed in penetrating media of high refractive index without damage.

In a normal sea urchin egg before and just after fertilisation, the only structure which shows an appreciable birefringence is the egg surface or cortex. Runnström, Monné and Broman [15] showed that this birefringence was negative with respect to the tangent to the surface (equivalent to positive with respect to the radius). It was also found by Monroy [12] that this birefringence diminished on fertilisation.

This birefringence is a combination of form and intrinsic birefringence, and in order to separate them one must immerse the eggs in a medium of high refractive index. Mitchison and Swann [11] made some observations on eggs which had been fixed in formalin and then immersed in a solution of potassium mercuric iodide with a refractive index about that of protein. In these eggs the negative birefringence of the cortex was stronger than it was in normal eggs in sea water. Also, under these conditions, fertilised eggs showed less birefringence than unfertilised eggs.

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[1] Presented before the Symposium on the Physiology of Fertilization, Palermo, April 26–30, 1955.

These results show that there are two components of the normal birefringence. First an intrinsic birefringence which indicates a predominantly radial arrangement of the *molecular* structure, and secondly a form birefringence which indicates a tangential arrangement of the *micelles*. Both the form and the intrinsic birefringence diminish on fertilisation.

The difficult thing, however, in work with polarised light is to determine the details of the structure which causes the birefringence, and, in particular, what substances are present in this structure. In the case of the sea urchin egg, there is some disagreement about the interpretation of the polarised light evidence.

Let us assume for the moment that the birefingence is present throughout the cortical layer, and that this layer is about 1.5 μ thick. Let us also assume that there is, at the outer edge of the cortex, a thin bimolecular layer of lipids about 40 Å thick. There is good evidence for the presence of such a layer from the permeability and electrical properties of the membrane. This layer would have a weak negative (tangential) intrinsic birefringence. There might also be a few layers of protein beneath this membrane arranged tangentially, and having a positive form and intrinsic birefringence.

Beneath these thin surface layers lies the majority of the cortex which appears from its mechanical properties to be a gelated structure, but is indistinguishable from the cytoplasm when observed with an ordinary microscope. There are two views about the sub-microscopic structure of the cortex. One view is that the cortex or, at any rate, the outer layers of the cortex, are composed of a number of lipid-protein layers which are repetitions of the surface layer i.e. lipids arranged radially and proteins arranged tangentially. Such a structure would be analogous to the myelin sheath of nerve fibres. The lipids would give the negative intrinsic birefringence of the surface, while the proteins would give the positive form birefringence. The proteins would also have a positive intrinsic birefringence, but this would be weaker than that due to the lipids. The other view is one put forward by Mitchison [8] that the majority of the cortical birefringence is due to the proteins only. The proteins are assumed to be in a looped or coiled arrangement. In this case, the micellar axes are lying tangentially and would give the positive form birefringence, but the molecular axes are lying radially and would therefore produce a negative intrinsic birefringence.

There are two pieces of evidence which support the latter view of the structure of the cortex. One of them comes from the optical properties of other cell membranes. The most obvious thing about the birefringence of the other cell membranes that have been examined is that they all show the same combination of positive form and negative intrinsic birefringence which appears in the sea urchin egg [8]. In most cases (e.g. amoebae, nerve cells, or spermatocytes) we are as ignorant about the composition of the membrane as we are in sea urchin eggs. But there is one exception—the red cell ghost. It is doubtful whether the red cell is a representative cell, but its membrane—the ghost— does have the same polarised light properties as other cell membranes; and it has the great advantage that it is only the cell membrane which can be got in quantity and analysed for its chemical constituents. Mitchison [9] carried out a detailed polarised light analysis of the red cell ghost, and one of the main things that emerged was that the birefringent membrane was about 5000 Å thick. Now it is known from chemical analysis that there is only enough lipid in the ghost to form a bimolecular layer at the surface about 40 Å thick. If we allow the presence of this surface layer of lipid—and it appears to be there from other evidence—then it follows that the birefringence in the rest of the membrane must be due to the protein which is the only other main constituent of the ghost. From the work on the red cell ghost we get a picture of the cell membrane with a thin surface layer of lipid which acts as the permeability barrier, and a much thicker layer of dilute protein gel which provides the main mechanical strength of the membrane, and which also produces most of the optical effects. This picture is supported by the second piece of evidence, which comes from the recent work with the electron microscope on thin sections of cells (e.g. [5, 16, 17]). All the cells which have been examined so far, including the sea urchin egg, show a very thin cell membrane, less than 200 Å thick. This presumably represents the lipid layer at the surface which acts as the permeability barrier. There is no sign of any multi-layered structure similar to what has been found in the myelin sheath. It may be thought that there should be signs of the looped protein structure that has been suggested for the cortex. But it is doubtful if a structure of this sort would be detectable with the electron microscope. The cortex is not a dense structure (in the case of the red cell ghost the concentration of protein must be only about 1–2 per cent), and it would differ from the cytoplasm in sea urchin eggs only in the fact that it is gelated, and in the details of the molecular arrangements of the proteins. In any case, all the electron microscope sections have been fixed and "stained" with osmic acid which would be expected to show up lipid structure but not protein structure. However, as electron microscope technique develops, we may get better methods for showing up protein structure which will clear up this point.

The structure of the sea urchin membrane would be clearer if two things could be discovered. The first one is the chemical composition of the membrane. This would indicate how much protein and lipid were present and whether the quantities were sufficient for the postulated structures. The difficulty is, of course, to find a method of separating the cell membranes in numbers sufficient for chemical analysis. Chambers [2] has separated the cortex by microdissection in the case of starfish eggs, but this has not been done with sea urchin eggs. In any case it would be a lengthy process to get enough membranes by this method. What is really wanted is a method similar to that used by Mazia and Dan [4] for separating the mitotic apparatus of cleaving eggs.

The second point is the thickness of the layer that is responsible for the birefringence at the surface of sea urchin eggs. It has been assumed so far that the whole of the cortical layer is birefringent, and this has been the assumption of most of the previous workers in this field. But it is possible that the birefringence might be caused only by the very thin surface layer (the lipid permeability barrier) together with a layer of associated protein only of a few molecules in thickness. It would be well worthwhile investigating this point, but the technique is difficult since full allowance has to be made for diffraction effects; and it would probably be necessary to work with sections.

Minganti [6] has recently published an interesting paper on the structure of the egg surface in which he makes two objections to the cortical structure suggested by Mitchison [8]. He finds that proteolytic enzymes do not affect the birefringence of the surface, whereas the phospholipase in bee venom reduces it. He suggests therefore that the birefringence is due to lipids or phospholipids rather than to proteins. A proteolytic enzyme would, however, not be expected to affect the surface since it would have to get through the surface lipid layer before it could act on the protein structure beneath. The phospholipase, on the other hand, might well act indirectly on the proteins. The proteins in the cortex are presumably stabilised in their gelated and ordered state by the presence of some structure. It seems very likely that this structure is, in fact, the lipid layer at the surface; so, if this lipid layer is disorganised by an enzyme, the effect will spread to the protein layer beneath.

The other point in Minganti's paper is that he finds that oocytes which have been slightly stretched show anomalous birefringence colours when stained with Nile Blue Sulphate. These colours imply an orientation of the dye molecules, and he believes that the dye must be bound in some ordered lipid structure. It may be that the dye molecules are present in the bimolecular lipid layer at the surface, but it is unwise to place much weight on this until we have a clearer idea of the reactions of dyes with biological molecules. Nile Blue Sulphate is certainly used as lipid stain, but it has also been used as general vital stain (references in [3]). In any case, anomalous birefringence at a cell surface can occur with other dyes which are not lipid stains (e.g. Toluidine Blue), as was found with amoebae by Mitchison [7].

However, it would be worth investigating the whole question of anomalous birefringence colours with dyes. This technique has the great advantage over other kinds of polarised light microscopy that the presence of the colours serves to identify the molecule that is causing the birefringence — which is normally impossible with a polarising microscope. Also, by a comparison of the anomalous birefringence with the dichroism it is possible to tell which way the dye molecule is lying. If there was more information about the attachment of dyes, this might be a very valuable tool for investigating submicroscopic structure.

As was mentioned earlier, the birefringence of the surface drops at fertilisation and does not reappear for some minutes. Since this is a drop in both form and intrinsic birefringence, it seems reasonable to assume that this is due to a disorientation of the molecules. One is naturally tempted to link this disorientation with the elevation of the fertilisation membrane, and to suggest a mechanism similar to that put forward by Runnström, Monné and Broman [15] and by Minganti [6]. If a membrane with radially arranged molecules is disoriented, it will expand in area and contract in thickness. An active expansion, similar to that which Swann and Mitchison [8] have suggested as the mechanism of cleavage, has in fact been found by Chambers [2] in the fertilisation membrane of starfish. The protein structure in the membrane suggested by Mitchison [8] fits in with this idea since it provides just the required radial orientation of the molecules. But there are two difficulties which should be pointed out. First, only a part of the cortex can be involved, because of the relative thickness of the cortex and the fertilisation membrane. The fertilisation membrane is about 500 Å thick in the wet state [10], so it can only have come from a layer about 1000 Å thick round the egg—which is only 6 per cent of the total thickness of the cortex. Secondly, if the fertilisation membrane comes from a part of the cortex, it should carry on its outside the original bimolecular lipid layer. But there is no sign of such a layer.

One other point which should be made about the birefringence changes at fertilisation is that they are very similar to those that take place later at cleavage. At cleavage there is also a fall in birefringence followed by a rise. This is true of the other optical changes mentioned below, and also of a number of physiological effects.

The other important optical property which changes on fertilisation is the scattering of light by the egg surface. The best-known change in light scattering occurs with dark-ground illumination, and was first observed by Runnström [14]. The cortex of the egg becomes markedly brighter on fertilisation, and Rothschild and Swann [13] found that this increase in light scattering spreads round the surface of the egg from the point of sperm entry, taking about 20 seconds.

Under dark-ground conditions the microscope detects light that has been scattered at about 60° from the direction of the incident beam. It is also possible to pick up light which has been scattered at larger angles by using forms of overhead illumination. With a Leitz Ultropak, the microscope receives light scattered at about 120°, and with a vertical incident illuminator it receives light scattered back at 180°. Under these conditions, the cortex of the unfertilised egg is bright, and the scattering diminishes at fertilisation —behaving in the opposite way to dark-ground [11].

It can be seen that what occurs at fertilisation is an increase of the light scattered at small angles and a decrease of the light scattered at large angles. These changes are usually interpreted as an indication of an increase in particle size.

Changes in the light scattering properties are a subject that might well be worth following up, since it is a standard method in physical chemistry for investigating molecular behaviour. It would be necessary to build an apparatus which could measure the intensity and degree of polarisation of light scattered at different angles.

It is worth mentioning one other form of special microscope which has been used on sea urchin eggs—the interference microscope [11]. So far, it has not been possible to use it to any effect on the cell surface, but has yielded some interesting information about sea urchin eggs. It is very sensitive to the presence of small concentrations of material, and it has shown that there is a material present in the perivitelline space (presumably a colloid) at a concentration of about 0.07 per cent in sea water. It has also provided the negative evidence that the concentration of material present in the jelly cannot exceed one part in 20,000. Finally it has shown that the dry thickness of the fertilisation membrane is about 160 Å.

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MICROSCOPIC AND SUBMICROSCOPIC CHANGES OF THE CYTOPLASM[1]

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When a sea urchin egg is fertilised, the main visible changes are those that take place in the cortex and at the surface of the egg. Under the low power of the microscope the most obvious effects are a temporary wrinkling of the surface and the elevation of the fertilization membrane.

The wrinkling is a well-known phenomenon, but surprisingly little is known about it. It looks as if the surface layers were expanding and getting too large for the available surface of the egg, so it is natural to associate it with the expansion of the fertilisation membrane. It does not appear to be due to a contraction of the egg volume, since there is little or no detectable change in the volume on fertilisation.

One of the important problems about the fertilisation membrane is where it comes from. Does it come directly from the egg cortex, or does it come from a vitelline membrane? A vitelline membrane can be defined as a zone or membrane which can be distinguished optically at the outer surface of an unfertilised egg, and which gives rise to the fertilisation membrane when the egg is fertilised or activated. Now it has been shown on a number of occasions that a thin membrane, which has been called the vitelline membrane, can be separated from the surface of unfertilised eggs either by crushing the eggs [6], by shrinking them in hypertonic solutions [16], or by microdissection [1]. These experiments, however, only show that a membrane *can* be produced at the surface of an unfertilised egg, and not that there is normally a vitelline membrane present in the undamaged egg. It must be remembered, also, that these mechanical treatments can activate eggs, so it is quite possible that what were called vitelline membranes were in reality fertilisation membranes.

The critical question is whether or not a vitelline membrane can be seen at the surface of a normal unfertilised egg. The early evidence is conflicting —some workers say that they can see a thin surface membrane [6, 18], others that they cannot [1, 4]. It may of course be easier to see in some species of sea urchin than in others, but it is easy to suspect observations like those of Kite [8] who found a vitelline membrane 1.9μ thick which was only visible when the condenser was stopped down. This is just the optical condition which produces prominent diffraction rings at the egg surface.

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The edges of living sea urchin eggs were examined with a high power water-immersion objective by Mitchison [10]. This gave the best possible optical conditions, and showed as much as is likely to be seen with an ordinary light microscope. Under these conditions, the edge of an unfertilised egg showed a series of dark rings diminishing in thickness and contrast from the inside outwards. At first sight the inner pair of rings looked as if they might define the edges of a vitelline membrane. This, however, seems unlikely in view of the fact that a very similar appearance was given by a fertilised egg. It is also interesting to find that trypsin treatment did not affect the pattern at the edge of the unfertilised eggs, which suggests that it is not digesting away a visible membrane. The conclusion from these observations is not certain. Undoubtedly *most* of the rings are diffraction rings, and the resemblance between a fertilised and an unfertilised egg suggests that they are *all* diffraction rings, but too little is known about the effect of diffraction at the edge of a complex object like a sea urchin egg to be certain about this point.

It is clear that we shall not learn very much more about the nature or existence of the vitelline membrane from observations with an ordinary microscope, since it is too near the limit of resolution. It is obviously a matter for the electron microscope and thin sections. So far, the evidence seems to be against the presence of a vitelline membrane. Neither McCulloch [9] nor Hillier, Lansing and Rosenthal [7] have found any obvious vitelline membrane in thin sections. In *Arbacia* eggs there is a thin surface membrane with papillae on it, but, according to the paper of Hillier and his colleagues, these papillae are still present in the fertilised egg.

Turning now to the egg cortex, there are a number of changes which take place at fertilisation. One of the most interesting is the change which takes place in the cortical granules. These have been investigated by Motomura [14], and Runnström, Monné and Wicklund [17]. There has also been a recent paper by Endo [3] which has some very good photographs of the behaviour of these granules in Japanese sea urchins. According to Endo, there is a single layer of granules in the cortex of the unfertilised egg, and these granules are about 0.5–1μ in diameter. When the egg is fertilised these granules swell up or explode to about twice their previous size and are then extruded from the surface. They attach to the inner surface of the fertilisation membrane under normal conditions, and they are then believed to take part in the hardening of this membrane. This is a most interesting process and it would be valuable if more could be found out about the nature of the granules, and their reaction with the fertilisation membrane. The granules are very obvious in thin sections under the electron microscope and they show a definite internal structure [2].

Another observation made by Endo is that the explosion of the cortical granules starts at the point of sperm entry and then travels over the surface of the egg like a wave, reaching the other side in about 15 secs. This is strikingly similar to the change in the dark-ground appearance of the cortex which has been observed by Rothschild and Swann [15], and it suggests that the optical effect may be due either directly to the change in granules or to a submicroscopic change which also explodes the granules.

The eggs of *Arbacia*, and some other sea urchins, have pigment granules in the cortex which are different from the cortical granules. These also change their distribution on fertilisation. In the unfertilised egg, they are scattered throughout the cortex and cytoplasm, but at fertilisation the majority of them move into the cortex [5].

There appears to be no detectable change in the thickness of the cortex at fertilisation. Mitchison [10] made observations on centrifuged eggs, where the cortex shows up clearly, and could find no significant difference in the thickness of the cortex before and after fertilisation. In sectioned eggs the thickness of the cortex was 1.15–1.35μ, and in living eggs it was about 2μ. It is reasonable to take a figure of 1.5μ as an average. This method of measuring thickness is unfortunately not sensitive enough to tell us whether or not a layer of about 1000 Å has been lifted off the cortex to form the fertilisation membrane.

We have considered so far the obvious visible changes that take place in the cortex at fertilisation, but there are also changes going on in the submicroscopic structure. Some of these are shown by optical changes [11]. They can be summarised as a decrease in the birefringence which implies a disorientation, and a decrease in the large angle scattering which implies an increase in molecular or particle size.

There is, however, one aspect of structure which should be mentioned. This is the rigidity of the cortex, which is presumably one aspect of its submicroscopic arrangement. Mitchison and Swann [12] have devised an instrument for measuring the rigidity of the cell surface, which they have called the "cell elastimeter". In essence it is very simple, and consists of a micropipette, about half the diameter of a sea urchin egg, which is connected to a movable reservoir of water. The pipette is brought near an egg and the reservoir is then lowered so as to suck the egg onto the end of the pipette. As the negative pressure or suction is increased a bulge is lifted from the egg surface, and the size of this bulge is then measured for increasing pressures. If these two measurements are plotted on a graph, a straight line is produced, and it can be shown from experiments with models that the slope of this line is proportional to Young's Modulus—or, in less exact terms, the rigidity or stiffness of the cell membrane. It is possible by this method to measure the membrane stiffness of a sea urchin egg in about one minute.

The difficulty of measuring rigidity at fertilisation is that the elevation of the fertilisation membrane interferes with the readings. As soon as a normal egg is fertilised the stiffness rises to a high value, and the effect of the cell membrane cannot be separated from that of the fertilisation membrane, which is much stiffer than the fertilised egg when it is fully formed. The only satisfactory solution is to use eggs where the elevation of the membrane has been inhibited by treatment with trypsin. In these eggs, there is a sharp rise in stiffness which reaches a peak within the first three minutes after fertilisation [13]. In the next five minutes, the stiffness falls back to a value slightly below that for normal eggs at the sperm aster stage. The precise position of the peak is uncertain since it is impossible to make instantaneous readings at fertilisation.

The difficulty about these experiments is that it is uncertain what trypsin is doing to the egg surface. It certainly has a drastic effect on the rigidity of unfertilised eggs, which drops to $^1/_4$ of the value of normal unfertilised eggs. It can only be hoped that the stiffness changes found at fertilisation in the trypsin-treated eggs are representative of the changes which take place in normal eggs.

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SOME EXPERIMENTS CONCERNING THE CHEMICAL MECHANISMS OF THE ACTIVATION OF THE SEA URCHIN EGG[1]

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It is the purpose of the present report to give an account of some experiments which, it is believed, may have some bearing on the problem of the early reactions involved in the activation of the sea urchin egg.

The starting point of these experiments was the observation that upon fertilization or parthenogenetic activation the orderly submicroscopic structure of the cortical layer of the egg is lost [9, 13]. Although there is still much uncertainty as to the actual structure of the cortical layer [8] it seems safe to assume it to be a lipoprotein system [10, 14]. It was suggested that upon activation the bond between lipids and proteins in the cortical layer of the egg is somewhat weakened or broken due to some factor introduced by the sperm [10].

There is some indirect evidence, dating back to the experiments of Herlant [4], that upon fertilization the egg cortex becomes impoverished of its phospholipid content. In fact the sea urchin egg has been found to become more resistant to the action of saponin, taurocholate and lecithinase during the first few minutes following fertilization [4, 14, 15, 12]. It seems interesting to recall here that in the eggs of the Polychaete worm, *Hydroides norvegica*, and of the Tunicate, *Phallusia mamillata*, an opposite type of reaction occurs. In the eggs of these animals fertilization indeed brings about increased sensitivity to the cytolytic action of taurocholate [12].

With the aim of obtaining some insight in the nature of the reactions that may occur between the fertilizing spermatozoon and the cortical layer of the egg at fertilization, a model experiment has been devised in which sea urchin sperm were made to react with a lipoprotein. The lipoprotein of the hen's egg yolk (lipovitellin [2]) was chosen for this purpose [6, 11].

When the sperm–lipovitellin mixture was extracted with cold ether, an increase of free phospholipids was found to occur during the first 10–15 minutes. It appears thus that the sperm are able to cause a release of free phospholipids from a lipoprotein. This reaction, however, soon proved to be more complex. In fact it was found that after a first rise, the amount of free phospholipids begins to decrease, thus suggesting that phospholipids are possibly broken down to ether-insoluble products. At the same time a hemolytic activity was detected in the mixture. After reaching a maximum this activity also undergoes a decrease. In control suspensions of sea urchin sperm in sea water a rapid decline of the free phospholipid content was found to take place (Fig. 1).

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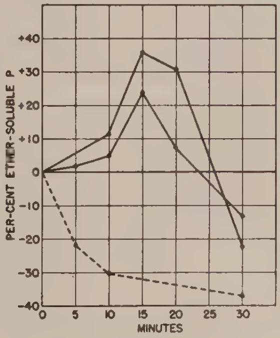

Change in the ether-soluble P (free phospholipids) when *Arbacia* sperm are incubated with a solution of lipovitellin (O——O) and in a control, i. e. sperm in sea-water (▲---▲).

It thus seems that when sea urchin sperm are made to react with a lipoprotein, phospholipids are first released which thereafter undergo a breakdown through the activity of sperm phospholipases. From the sequence of the events it looks as if two phospholipases were at work causing the removal of the two fatty acid residues from the phospholipid molecule. The removal of the first changes the phospholipid molecule into a lysophosphatide (appearance of the hemolytic activity) while the removal of the second leads to a non-hemolytic product, e.g. glyceryl–phosphoryl–choline. The fact that the latter is ether-insoluble and water-soluble, accounts for the decrease of the ether-soluble phosphorus.

These observations have led us to speculate about the nature of the reactions that may occur at the fertilization of the sea urchin egg. When the fertilizing spermatozoon reacts with the cortical layer of the egg, from the point of its entrance, a reaction is started which rapidly propagates all over the surface of the egg as well as to the whole cytoplasm. How this propagation occurs is obscure at present. We assume it to consist in the splitting of the lipoprotein complex of the cortical layer of the egg followed by the stepwise enzymatic breakdown of the released phospholipids and the transient appearance of a cytolytic activity. It is also hypothetically assumed that the lysophosphatides are responsible for the first reaction leading to the activation of the egg, although no evidence is at present available as to the mechanism by which lysophosphatides may be operative in this process, nor as to their point of attack. The appearance of the lysophosphatides must be a very transient occurrence and the reactions that have been found to occur during some minutes in our model system must necessarily be speeded up considerably in the natural process. Furthermore, lysophosphatides will appear in very minute amounts. For these two reasons any search for them may prove very deceptive.

The fact that eggs may be activated parthenogenetically indicates that lysophosphatides may simply be instrumental in releasing another reaction which may, however, be initiated by a variety of agents having in common the ability of acting as denaturing agents of proteins. For example, it may be that the crucial step of activation is the release of a substance "X" from its bound state. This hypothetical chain of events may be summarized as follows:

$$\begin{tikzcd}
\text{Sperm} \arrow[r] & \text{Cortical lipoprotein} \arrow[r] & \text{Lysophosphatides} \arrow[r] & \text{Bound ``X''} \arrow[r] & \text{Free ``X''} \\
 & & & \text{Parthenogenetic agents} \arrow[u] &
\end{tikzcd}$$

An alternative possibility is that the lysophosphatides may be formed in the egg by the action of the activating agent.

It has been shown that some of the proteins of the egg undergo considerable changes immediately upon fertilization. Recent evidence from this Laboratory suggests that a group of proteins undergoes some sort of internal rearrangement as indicated by their response to treatment with urea [1]. Proteins of the fertilized eggs seem indeed to be more easily denatured by urea than those of the unfertilized eggs. Determinations of the intrinsic viscosity of this same fraction before and after fertilization have, on the other hand, yielded the same value: hence it does not seem that fertilization may bring about any considerable change of shape of the molecules of this fraction. The affinity of proteins for Ca^{++} also appears to decrease, as indicated already by the investigations of Mazia [7]. Our colleague D'Amelio [4] has recently shown that an isotonic solution of $CaCl_2$ injected into the egg does not mix with the cytoplasm, but forms a vesicle well delimited by a thick wall. This, however, disappears as soon as the egg is fertilized and the cytoplasm re-acquires its homogeneous appearance.

How these changes may be brought about is unknown at present. It must be mentioned, however, that during the first few minutes after fertilization activation of proteolytic enzymes has been found to take place [5]. Experiments in progress in our laboratory indicate that these enzymes are localized in the mitochondria. It is likely that they may be instrumental in the above-mentioned changes in the proteins and they probably play some role in fertilization. This is indicated also by some other experiments in progress in this Laboratory that show that in butyric acid activated eggs (which are known to undergo cytolysis after a number of monaster cycles not followed by cleavage) a continuous increase of the proteolytic activity takes place. Furthermore in these eggs proteolytic enzymes are also found in the cytoplasm besides those in the mitochondria.

However, it is at present very hard to visualize how these changes are linked to the first ones which I assume to be the sparking reaction of the activation.

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RESPONSE OF UNFERTILIZED SEA URCHIN EGGS TO ANTISERUM

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Serological methods are often used for studies of surface structures of cells such as bacteria, blood cells and protozoa. It therefore seemed reasonable to start a similar investigation on sex cells which are known to contain surface elements of high specificity [29, 31, 46]. We also know that one or more reactions of high specificity are involved in the process of fertilization and the possibility of a parallelism between certain of these interactions and antigen–antibody reactions has previously been pointed out [44].

The surface layers of the sea urchin egg are relatively well known from microscopical, chemical and physiological studies [32]. This cell thus seems to offer a suitable object for a serological approach. It was hoped that a study of this sort would not only contribute to our knowledge of the molecular structure of the egg surface but also throw some light on the mechanism of sperm attachment and the initiation of egg activation.

In 1954 the author showed that the treatment of unfertilized sea urchin eggs with homologous rabbit antiserum may lead to a number of drastic changes, involving membrane elevation and nuclear activation [25]. Later it was also observed that the fertilization rate was greatly decreased in eggs pretreated with homologous antiserum under conditions which did not bring about the visible changes mentioned above [26]. By absorption experiments it could be proved that the decrease in fertilizability was caused by a primary effect of the antiserum on the eggs and not on the sperm.

In a number of forthcoming publications a fuller account of the different antiserum effects will be given. The aim of this first paper is to describe the action of antiserum on unfertilized eggs, to show the conditions which must prevail in order to obtain a response in the eggs and to describe the specificity of the reactions.

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[1] Presented before the Symposium on the Physiology of Fertilization, Palermo April 23–26, 1955.

A few details concerning experiments carried out during May–July 1955 have been added later.

MATERIAL AND METHODS

The experiments were performed in 1954 and 1955 during the months of February–July at the Zoological Station of Naples, Italy. The most thoroughly studied sea urchin species was *Paracentrotus lividus*. To a minor extent eggs from *Psammechinus microtuberculatus*, *Arbacia lixula* and *Sphaerechinus granularis* were also used. The eggs were collected and washed according to standard procedures previously described (e.g. [13]).

Antisera were obtained by injecting rabbits, weighing about 3.5 kg, with sea urchin material. Saline suspensions of lyophilized eggs or concentrated, previously deep frozen egg homogenates were used for the injections. A few antisera were prepared against the isolated substance of the jelly layer of *Paracentrotus* eggs. Isolation and purification of the jelly substance were performed according to Vasseur [53].

Blood samples for control purposes were taken from all rabbits before the injections were started. Different methods were used for injecting the antigens. In most cases one intravenous injection was given, followed by a rest period of 3 weeks and then a series of 10 subcutaneous injections on alternating days (presensitization method). The rabbits were bled to death one week after the last injection. In a few cases the rabbits obtained 2 intravenous injections a week for a period of 4 weeks. Increasing amounts of antigen were injected and each intravenous injection was preceded by a subcutaneous one the day before. In a few additional cases the antisera were prepared by means of Freund's adjuvant techniques as described by Cohn [4].

Generally 3–5 rabbits were given injections with the same preparations and the sera from these animals were pooled. They were dialyzed against sea water, Seitz filtered and stored at −10°C until use. If necessary the pH was adjusted to 7.8 by addition of NaOH. Apart from the serum samples mentioned above, 3 pooled sera, each from 5 uninjected rabbits, were used as controls. The control sera were subjected to the same treatment as the antisera. Further details concerning the sera referred to in this paper are given in Table IX and in the text below.

The eggs were exposed to the sera in Boveri dishes. Usually the total volume of the reaction mixture was kept constant (1 or 2 ml). Varying amounts of eggs were used and the final concentration of the sera was varied from 5–80 per cent by volume. The dishes were covered to prevent evaporation and very gently rotated by means of a mechanical device in order to assure complete mixing. The experiments took place in a constant temperature room at about 20°C throughout the whole season. When counts of the number of affected eggs were made, the dishes were placed directly under the microscope, and about 250 eggs were counted in each bowl. If not otherwise stated the experiments were set up in duplicate. The percentage figures given below are average values from the duplicate experiments. Duplicates differing by more than 10 per cent were rare and are not included with the data given in this paper. Further experimental details will be given below.

DESCRIPTION OF THE ANTISERUM ACTION

Precipitation of the jelly layer.—When mature, unfertilized eggs were exposed to homologous antiserum, a precipitation of the jelly layer around the eggs was often obtained (cf. [25], Figs. 1, 2, 4). As will be shown below, the jelly-precipitating potency of the antisera varied considerably. When it was low the action could be enhanced by the addition of isotonic sucrose solution to the reaction mixture as described previously [25]. In these cases the jelly layer became invisible again if the eggs after completion of the experiment were transferred to sea water. In the case of the more potent antisera no sucrose addition was necessary. Usually a precipitation ring on the outer surface of the jelly layer became visible within the first 10 minutes of exposure. Little by little the jelly layer was contracted, thereby often forming a narrow rim near the egg surface. This jelly precipitation was irreversible and the layer remained visible and unchanged even after removal of the serum and transfer of the eggs to sea water. Eggs with precipitated jelly layer were often agglutinated.

Table I

Effect of egg number on jelly precipitation in *Paracentrotus lividus.*

500, 5000 and 50,000 eggs from the same female, antiserum in varying amounts, total volume of reaction mixture 1 ml. Observation after 120 minutes at 20°C. Figures based on counts of 250, 500 and 2000 eggs respectively.

0 = No visible response, + = Weak jelly precipitation, + + = Moderate jelly precipitation, + + + = Strong jelly precipitation.

Per cent antiserum	Response		
	500 eggs	5000 eggs	50,000 eggs
80	~100 p.c. (+ + +)	~30 p.c. (+)	0
40	~70 p.c. (+ +)	0	0
20	~10 p.c. (+)	0	0

In order to get a visible manifestation of the antiserum action, the number of the eggs in a given amount of serum must not be too high and/or the antiserum concentration must not be too low. Table I gives the results from a typical experiment in which both the egg number and the antiserum concentration were varied.

Naturally, the lack of any visible manifestation in an experiment of this kind does not mean that a reaction has not occurred. Thus, when 100,000 eggs or more were allowed to stand in 1 or 2 ml antiserum and the serum after the removal of the eggs was tested with a new lot of 500–1000 eggs, usually no visible jelly-precipitation occurred, even when a correspondingly diluted control with fresh antiserum gave a strong response. Only occasionally were 2 absorptions of this type needed in order to obtain a complete elimination from the antiserum of the jelly-precipitating agent.

When pretreating the eggs with crystalline trypsin at concentrations which did not dissolve the jelly layer (Armour Co., final concentration $5 \cdot 10^{-4}$–$1 \cdot 10^{-3}$ per cent, 10–20 minutes) the precipitability of the jelly layer was not affected. Furthermore, only in 1 out of 9 experiments was the precipitability of the layer noticeably reduced by a pretreatment of the eggs with IO_4^- (final concentration of $NaIO_4$ $5 \cdot 10^{-6}$–$5 \cdot 10^{-5}$ *M*, 15–30 minutes). However, periodate treatment led to a marked crenation of the otherwise smooth jelly surface. Treatment of the eggs with acidified sea water (pH 3) did not change the reactivity of the jelly layer. Such a treatment generally dissolved the jelly layer and left most of the eggs denuded. However, eggs may sometimes be found from which the jelly layer had not been removed. When these were exposed to antiserum normal response was obtained. On the other hand, weakened response could sometimes be observed when the eggs after shedding were allowed to stand for 5 hours or more before exposure to antiserum. Prolonged washing with sea water often gave similar results.

The reactivity of the eggs is rather variable. With *Paracentrotus,* using an antiserum of good potency and standard conditions (50 per cent antiserum and 500 eggs in a reaction mixture of 1 ml, observation after 2 hours at 20°C) a positive response was obtained on the eggs from about 50 per cent of the females. The results summarized in Table VI serve as an illustration. Usually 5–10 females were tested individually in the same experiment. The experiments were run over a period of 3 months. As can be seen from Table VI, the eggs from about half of the females were visibly affected when exposed to antiserum J-2. Among the responding females the reactions varied a great deal in strength, as illustrated by the typical data given in Table VII.

Weak or lacking response could sometimes be overcome by changing the experimental conditions in a more favorable direction, e.g., by decreasing the number of eggs and/or increasing the amount of antiserum in a reaction mixture, or by the addition of sucrose solution. It must be emphasized, however, that the possibilities of doing so were very limited when lack of response had been found in an experiment with one of the potent antisera used under standard conditions as defined above. In fact, females were always found whose eggs did not react at all, no matter how favorable the experimental conditions, and even among the eggs from well responding females more or less completely inert jelly layers occurred.

Upon a closer examination of the response data it was soon learned that the precipitability of the jelly layer varied considerably with the season of the year. Thus, with *Paracentrotus,* a weak average response was obtained on the eggs from only 10–30 per cent of the females during the months of February–April. During May the qualitative and quantitative responses gradually increased, culminating with a period of optimal reactivity (strong reactions in the eggs from about 80 per cent of the females) during the first weeks of June. Almost identical results of this type were obtained in 1953, 1954 and 1955.

There undoubtedly exists a correlation between the reactivity of the jelly layer to antiserum and the state of maturity of the eggs. This also becomes apparent from the fact that the jelly layers almost invariably are of a very low reactivity when the percentage of oocytes among the eggs from a certain female is high. In *Paracentrotus* the jelly layer of oocytes was only occasionally precipitable with antiserum. During May and June almost no oocytes occurred among the eggs in the reaction mixtures. It should also be mentioned that a low jelly reactivity was often found when cytolysis occurred among the eggs from a certain female.

The results given here are based on observations made on the eggs of *Paracentrotus lividus.* They are also valid for the eggs of *Psammechinus microtuberculatus* and *Sphaerechinus granularis.* However, the jelly reactivity of the *Psammechinus* eggs was much weaker. It cannot yet be stated whether that was because of properties inherent in their jelly layers or because of a low jelly precipitating potency of the three antisera used. The reactivity of the *Psammechinus* eggs culminated in May at the end of the spawning season, while *Sphaerechinus* did not reach optimum reactivity until July.

The jelly layer of the eggs from *Arbacia lixula* behaved in an exceptional way. They were relatively often precipitated by heterologous antiserum and by control serum (cf. Table III). But even in these cases the specific jelly precipitation by homologous antiserum led to a contraction of the layer, while unspecific precipitation was accompanied by a strong swelling of the layer. The reactivity of the *Arbacia* eggs was at an optimum at the end of June and in July.

Wrinkling effect.—When the eggs were left in antiserum, after a certain time a wrinkling of the cell surface could be observed. This wrinkling gradually grew stronger. It always started at one or a few points where the surface was contracting. From there it spread radially over the egg (Fig. 1) until the entire surface was affected (Fig. 2). In normal eggs a relatively fine wrinkling developed. Eggs previously freed from their jelly layer by short exposure to acidified sea water at pH 5.2 or below were much more strongly affected.

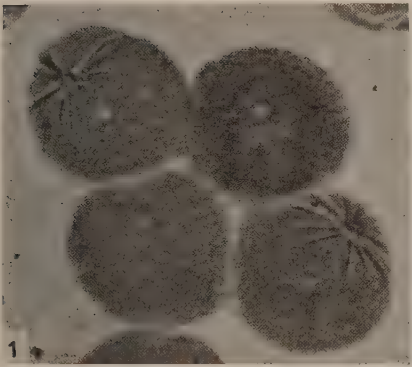


Fig. 1. Jelly-free eggs of *Paracentrotus lividus* after about 50 minutes exposure to homologous antiserum.

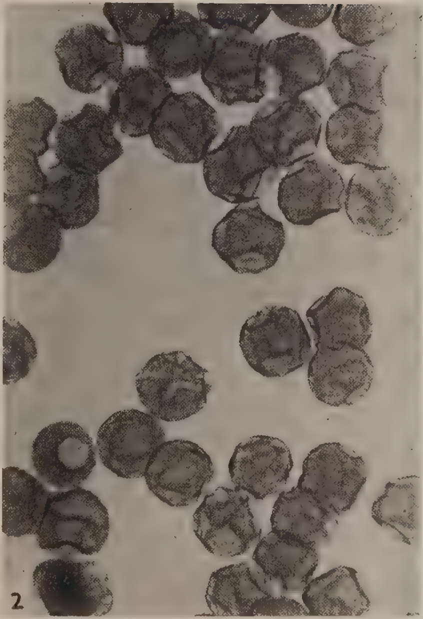


Fig. 2. Eggs from the same experiment as in Fig. 1 after about 100 minutes exposure to homologous antiserum.

Closer examination of the point where the reaction started showed that the surface was often visibly broken up and a little drop of coagulated cytoplasm could be seen outside the cell. When the antiserum treatment was interrupted before the eggs had been visibly damaged, they changed shape and regained their normal appearance, except for a small resting indentation at the point where the reaction had started.

The wrinkling response of the eggs to antiserum was rather slow. Fig. 3 gives the results of a typical experiment. During the first 50 minutes of the treatment most of the eggs looked completely normal. Then the number of the responding eggs increased rather quickly to a maximal value. As can be seen the visible response occurred faster in jelly-free eggs than in normal eggs. On the other hand, the final number of reacting eggs always seemed to be the same in both cases, even at low antiserum concentrations. However, classifying an egg with the jelly layer intact as affected or ont may have been subjected to a considerable experimental error.

Varying the number of eggs and/or the amount of antiserum in the reaction mixtures affected both the qualitative and quantitative responses. In the case of the jelly-free eggs, the classification of a cell as affected or not was relatively simple and the quantitative response could thus be recorded. Fig. 4 shows the results of an experiment where the percentage response has been plotted against the log of the antiserum concentration. S-shaped dosage-response curves were obtained. Egg numbers up to 10,000 usually gave > 90 per cent response with most of the antisera referred to in this study when sufficiently high antiserum concentrations were used. Increasing the number of the eggs still more resulted in a gradual decrease in the response. At egg numbers of 100,000 or more no microscopic change could be observed in the eggs, providing the amount of antiserum used was not too high. Previous treatment of a given volume of antiserum, e.g. 1 or 2 ml, with a large number of eggs abolished the wrinkling potency of the serum sample more or less completely. This means that an absorption had taken place as already described for the jelly-precipitating action.

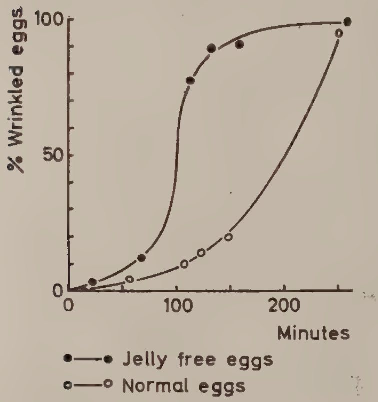


Fig. 3 (left). *Paracentrotus lividus*, 1000 eggs and 80 per cent antiserum, total volume of reaction mixture 1 ml.

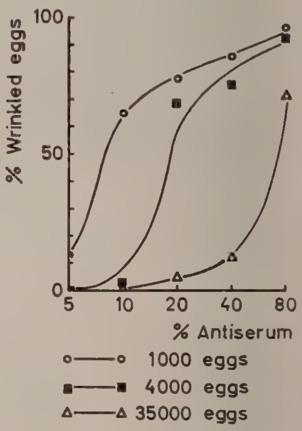


Fig. 4 (right). *Paracentrotus lividus*, varying amounts of jelly-free eggs and antiserum, total volume of reaction mixture 1 ml. Percentage of wrinkled eggs plotted against log percentage of antiserum.

Pretreatment of the eggs with trypsin as described above and exposure of the pretreated eggs to antiserum led to a complete inhibition of wrinkling. IO_4^- treatment (cf. above), on the other hand, caused a marked though apparently only qualitative enhancement of the antiserum action. Pretreatment of the eggs with acidified sea water at a pH as low as pH 3, standing after shedding and prolonged washing did not affect the response. Addition of sucrose resulted in a qualitatively weaker response.

The wrinkling effect of the antisera was the most predominant of all the effects described in this paper. With jelly-free eggs wrinkling could be obtained on most of the eggs from almost all females throughout the entire periods of experimentation (February–July), provided optimal experimental conditions were maintained. Considerable qualitative differences did, however, occur. The reactivity of eggs with their jelly layer intact was more variable. No clear cut correlation between precipitability of the jelly layer and wrinkling has yet been found.

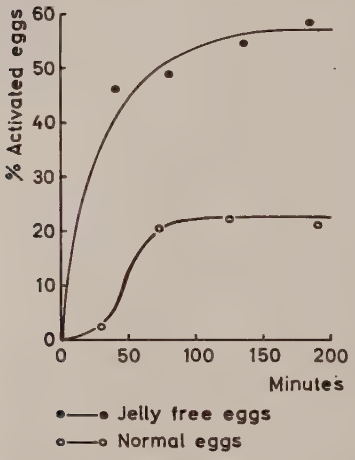
On exposure to antiserum, oocytes never responded in the way described above. Usually they underwent cytolysis and were often completely destroyed within the first 20 minutes of the treatment.

The findings reported in this section are valid for the eggs from all the sea urchin species investigated.

Activating effect.—In reality, the significance of the wrinkling response just described may be that the cortical layer of the affected eggs came into a state of preactivation [29, p. 286]. However, membrane formation was only rarely observed in typically wrinkled eggs and nuclear activation never occurred. These two phenomena were characteristic of still another activity which most of the antisera exerted on a certain percentage of the homologous egg material. The relative independence of wrinkling on the one hand and activation as defined by membrane elevation and nuclear activation on the other may justify, for the time being, a separate treatment of the two activities.

Usually about 20–30 minutes after the beginning of the antiserum treatment a membrane was elevated from the surface of the affected eggs. This membrane was very refractile and often remained attached to the surface at many points ([25], cf. Figs. 2, 3). Most of the cortical granules were probably not broken down. The surface changes were followed by a migration of the nucleus to the center of the cell, monaster formation and initiation of nuclear and cell division. In high antiserum concentrations the process usually became abnormal at this point. When activation had been brought about by antiserum in moderate concentration and the eggs in time had been transferred to sea water, a few cell divisions sometimes took place. But even then the appearance of the cleavage products formed was abnormal and development stopped at an early stage.

The manifestation of the activating effect of the antisera was significantly faster than that of the wrinkling. Fig. 5 gives a typical time–per cent response curve. Within certain limits, the time–response relationship was even in this case independent of the amounts of eggs and antiserum in the reaction mixtures. As in the case of the wrinkling effect the normal eggs responded slower than the jelly-free eggs. Even at high antiserum concentrations, the maximal number of responding normal eggs was generally lower than that of the jelly-free eggs. This is also illustrated by the data given in Fig. 6, where the per cent response from a typical experiment has been plotted against the antiserum concentration.



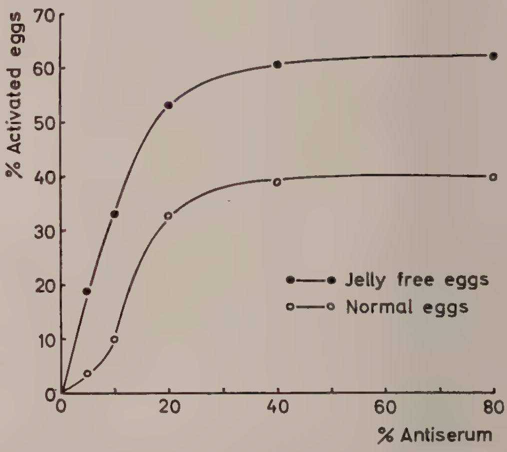


Fig. 5 (left). *Paracentrotus lividus*, 500 eggs and 80 per cent antiserum, total volume of reaction mixture 1 ml.

Fig. 6 (right). *Paracentrotus lividus*, 500 eggs and varying amounts of antiserum, total volume of reaction mixture 1 ml.

The inhibition of activation by the presence of the jelly layer around

TABLE II

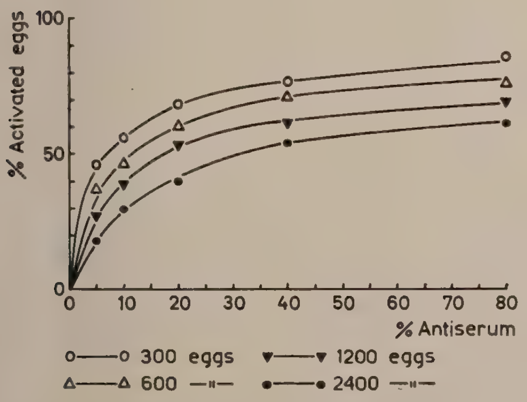
Activation of normal and jelly-free eggs from 10 different females of *Paracentrotus lividus*. 500 eggs, 50 per cent antiserum, total volume of reaction mixture 1 ml. Observation after 180 minutes at 20°C.

Female No.	Per cent activated eggs									
	1	2	3	4	5	6	7	8	9	10
Normal eggs	0	7	56	36	3	25	1	3	20	11
Jelly-free eggs	10.5	27	58	55	16.5	28	35	14	18	72

the eggs (cf. Figs. 5, 6) varied considerably. Table II shows the results of testing an antiserum on normal and jelly-free eggs from 10 different females. A further increase of the antiserum concentration in these cases did not bring about any increase in response. There seems to exist a rather complicated correlation between the precipitability of the jelly layer and activation. Activation was often strongly inhibited in eggs with a jelly layer easily precipitated by antiserum. Removal of the jelly layer previous to antiserum exposure then usually resulted in a greatly increased number of activated eggs.

Too many eggs in a given volume of antiserum led to a more or less complete suppression of activation. Absorption of the activating agent from the antiserum took place in this case as described above for the jelly-precipitating and wrinkling effects, although more than one absorption was usually necessary in order to remove the activating action entirely.

Within certain limits the number of responding eggs varied rather regularly with the concentration of the antiserum and inversely with the total number of the eggs in the reaction mixture. Thus, a straight line relationship was obtained when the per cent response of jelly-free eggs was plotted against the log of the egg/antiserum ratio. Figs. 7 and 8 give the results from an experiment of this kind, where both the egg numbers and the antiserum concentrations had been varied. A straight line can also be obtained based on the values from the jelly-free eggs in Fig. 6.



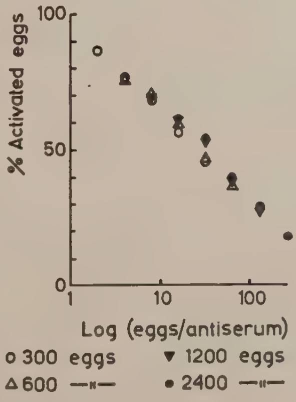


Fig. 7 (left). *Paracentrotus lividus*, varying amounts of jelly-free eggs and antiserum, total volume of reaction mixture 1 ml.

Fig. 8 (right). *Paracentrotus lividus*, percentage of activated eggs plotted against log (eggs/antiserum) calculated from the values in Fig. 7.

Pretreatment of the eggs with trypsin as described in the preceding sections did not decrease the number of the responding eggs. Activation took place though naturally without the formation of a membrane. Whether pretreatment with IO_4^- had any influence on the activating action of the antisera is not yet clear. Pretreatment of the eggs with acidified sea water at a pH below 5 strongly inhibited activation by antiserum. Addition of isotonic sucrose solution improved the response.

What has been said above about the sensitivity of the eggs to the jelly precipitating action of the antisera is to a certain degree also valid for their activating action. The data given in Table VI serve as an illustration. In that experiment, eggs from about 30 per cent of the 132 females studied could be activated by the action of both of the antisera used. With a few exceptions, it was the eggs from the same females which reacted in both sera. Table VII gives a more detailed picture of the results on the eggs from 5 females. This table also shows that no direct conclusions can be drawn concerning the possible correlation between jelly precipitability and sensitivity to the activating action of the antisera.

The antisera referred to above were both of a moderate activating potency. Increasing their concentration or using antisera of a higher potency would have led to an improvement of the response. However, the eggs from a certain percentage of females never responded even at optimal experimental conditions and among the eggs from well responding females usually some were found which could not be activated. As described for the jelly-precipitability, a certain positive correlation existed between the state of maturity of the eggs and their reactivity. This correlation was somewhat less marked than in the first named case.

The atypical response of oocytes has already been mentioned. Only in the case of *Psammechinus* was membrane formation sometimes observed in the oocytes.

The eggs from all the sea urchin species studied could be activated by the action of homologous antiserum. The optimal periods of reactivity were in May in the case of *Psammechinus microtuberculatus* and at the end of June and in July for *Arbacia lixula* and *Sphaerechinus granularis*.

SPECIFICITY

On eggs of *Paracentrotus, Psammechinus* and *Sphaerechinus* sera from untreated rabbits only rarely led to a precipitation of the jelly layer. As has been mentioned (see also Table III) the jelly layer of the eggs of *Arbacia* behaved somewhat differently. When occurring, the "non-specific" precipitation in eggs from all sea urchins investigated could easily be recognized as it always was accompanied by a swelling of the layer, caused by the proteins of the serum [37].

Wrinkling and activation have almost never been observed when using normal serum. More than 500 control tests have so far been made, using three different control sera (each a pool from 5 animals) and serum samples taken from the rabbits before they were injected with sea urchin material in order to produce antibodies. A combined treatment of jelly-free eggs with hypertonic acidified sea water and normal rabbit serum under certain experimental conditions led to membrane elevation. These membranes resembled normal fertilization membranes and were quite different from the refractile and granular antiserum membranes. Eggs activated in this manner always cytolyzed within one hour.

The observed inactivity of normal serum appears to be contradictory to the findings made by Loeb [19] and Robertson [27] as early as the beginning of the century. Activation of the eggs of the sea urchin *Echinocardium cordatum* by normal serum has also been reported by Runnström *et al.* [37]. It is not clear whether these discrepancies depend on differences in the preparation of the sera or differences in the sea urchin species investigated. Negative results with normal rabbit serum were also obtained by the author in experiments where the eggs previous to serum exposure had been treated with $SrCl_2$ as described by Robertson (l.c.). On the other hand, activation of *Paracentrotus* and *Sphaerechinus* eggs could be brought about with some samples of human serum. The activating action of these samples strictly followed the rules established by Robertson (l.c.). The membranes formed in this case had the character of normal fertilization membranes. Nuclear activation and cell division did not occur.

The species specificity of the reactions described in the preceding paragraphs can best be illustrated by the data given in Table III. The table contains the results from tests on the eggs from 30 different females, 10 from each of the 3 species *Paracentrotus lividus, Psammechinus microtuberculatus* and *Arbacia lixula*. Each female was tested separately but with homologous and heterologous antiserum and control serum in the same experiment. The sera were the same throughout the whole experiment. Females were chosen, the eggs of which responded fully when tested with homologous antiserum in preliminary tests. Jelly precipitability was recorded on normal eggs while the records of wrinkling and activation were made on lots from the same batch of eggs though previously freed from their jelly layer.

Table III

Ten females from each of the species *Paracentrotus lividus*, *Psammechinus microtuberculatus* and *Arbacia lixula* tested with homologous and heterologous antisera and normal rabbit serum. 500 eggs, 80 per cent antiserum, total volume of reaction mixture 1 ml. Observation after 180 minutes at 20°C. For further explanation see text.

Antiserum	Total number of females	Number of females with >10 per cent of eggs responding			Number of females with <10 per cent of eggs responding		
		Jelly ppt	Wrinkling	Activation	Jelly ppt	Wrinkling	Activation
Paracentrotus lividus females							
Paracentrotus	10	5	10	6	5	0	4
Psammechinus		0	0	1	1	1	5
Arbacia		0	0	0	0	0	4
Sphaerechinus		0	0	0	1	0	4
Normal serum		0	0	0	1	0	0
Psammechinus microtuberculatus females							
Paracentrotus	10	1	0	3	0	1	3
Psammechinus		4	9	6	1	1	4
Arbacia		0	0	0	0	0	5
Sphaerechinus		0	0	0	1	0	3
Normal serum		0	0	0	0	0	0
Arbacia lixula females							
Paracentrotus	10	6	0	0	2	0	2
Psammechinus		5	0	0	1	0	2
Arbacia		10	6	5	0	4	5
Sphaerechinus		6	0	0	2	0	2
Normal serum		5	0	0	3	0	0

As can be seen from the table, in the eggs from *Paracentrotus* and *Psammechinus*, jelly precipitation with heterologous antiserum occurred in only 4 out of 60 experiments (excluding the control serum experiments). Only in 1 out of these 4 cases was the number of responding eggs higher than 10 per cent. Homologous jelly-precipitation occurred in 15 out of 20 experiments and in 9 of them the response was high. The incomplete homologous response was caused by the relatively low reactivity of the *Psammechinus* jelly layer already mentioned. The different behaviour of the *Arbacia* jelly layer becomes apparent from the data showing heterologous responses in 22 out of 30 experiments.

Wrinkling occurred in 2 out of 90 experiments with heterologous antiserum. In 34 of these 90 activation was found, but only in 4 of them were more than 10 per cent of the eggs activated. In all 30 experiments with homologous antiserum wrinkling and activation occurred. More than 10 per cent of the eggs were wrinkled in 25 cases thereof and in 17 cases more than 10 per cent were activated.

Control serum was without wrinkling and activating effect in all 30 experiments. Precipitation of the jelly layer occurred mainly on the eggs of *Arbacia lixula*.

In Table IV the data from a specificity test are given in greater detail.

TABLE IV

Eggs from *Paracentrotus lividus, Psammechinus microtuberculatus* and *Arbacia lixula,* tested with homologous and heterologous antisera.

Mixture of eggs from 5 well responding females of each species. 1000 eggs, 50 per cent antiserum, total volume of reaction mixture 1 ml. Observation after 180 minutes at 20°C.

Antiserum	Per cent response in eggs from								
	Paracentrotus			*Psammechinus*			*Arbacia*		
	Jppt	Wr	Act	Jppt	Wr	Act	Jppt	Wr	Act
Paracentrotus	~80	>90	44	~30	0	10	~80[1]	0	0
Psammechinus	0	0	12	~30	>90	35	~80[1]	0	0
Arbacia	0	0	4	0	0	7	>90[2]	>90	25
Normal serum	0	0	0	0	0	0	~80	0	0
Sea water	0	0	0	0	0	0	0	0	0

Abbreviations: Jppt = Jelly precipitation
Wr = Wrinkling
Act = Activation.

[1] Jelly layer swelled.
[2] Jelly layer contracted.

The results given in Tables III and IV may be sufficient as proof of the high species specificity of the antisera used in this study. This species specificity was of course not absolute. Furthermore, their activating action appeared to be less specific than their wrinkling and jelly-precipitating action. However, it must be pointed out, that the antisera used in the experiments referred to above, were of a high activating but only of a moderate jelly-precipitating potency. Choosing strong jelly-precipitating sera would have given somewhat different results in favour of an increased heterologous jelly-precipitating action. Essentially different results from those reported here have, however, never been obtained. Moreover, a somewhat higher degree of cross reactivity was sometimes found between the species *Paracentrotus lividus* and *Psammechinus microtuberculatus* than between either of these two and *Arbacia lixula*. The significance of this difference has not yet been established.

Similar specificity rules seemed also to be valid for the eggs of *Sphaerechinus granularis*. So far, only a few experiments of this kind have been made on the eggs of this species and the results have therefore not been discussed here.

ANTISERUM REACTIVITY

The inactivity of normal rabbit serum and the species specificity of the antiserum action can be taken as definite proof that the effects reported are due to the presence of specific antibodies in the antisera. That does not necessarily exclude the possibility that normal serum components are involved in the reactions accompanying the primary combination of antigen and antibody at or near the surface of the egg cell.

Attempts to prove the concomitant action of normal serum factors have thus far been without success. Antiserum samples, heated to 56°C for 30 minutes for inactivation of complement did not seem to differ in their action from unheated control samples. In fact, the quantitative response of the eggs seemed to vary rather strictly with the amount of homologous antiserum in a reaction mixture. Table V serves as an illustration. The table shows the results from an experiment in which eggs from *Paracentrotus* had been treated with anti-*Paracentrotus*-serum, diluted with sea water or with control serum, giving a total serum concentration of 80 per cent. Control serum alone in corresponding concentrations had no effect. The percentage activated eggs obtained was almost identical in both cases, i.e., was independent of the

TABLE V

Activation of jelly-free eggs of *Paracentrotus lividus* exposed to antiserum diluted with sea water or control serum to a final serum concentration of 80 per cent.

1000 eggs, total volume of reaction mixture 1 ml. Observation after 180 minutes at 20°C. Control serum in 20, 50 and 80 per cent concentration without effect.

Concentration of antiserum in per cent	Per cent activation	
	Antiserum + sea water	Antiserum + control serum
20	32	30
50	69	72
80	70	69.5

nature of the diluent used. Similar results, also concerning jelly precipitation and wrinkling, were obtained in several experiments, using different control sera and different antisera. However, the entire problem of the possible co-operative action of unspecific serum components in evoking the antiserum effects described in this paper is still under investigation.

In the preceding sections reference has been made to antisera of "good jelly-precipitating" or "moderate activating potency" etc., thus intimating the existence of an antiserum variability. In the following paragraphs the nature of this variability, which in fact was considerable, will be briefly described.

Table VI serves as the first example. The results given there have already been considered in order to show the differences in the reactivity of the eggs. They may also be used as an illustration of the nature of the differences in antiserum reactivity. They emanate from tests on the eggs from 132 females, each tested separately with 2 different antisera. As can be seen, the anti-jelly-serum J-2 exerted a jelly-precipitating action on the eggs of almost twice as many females as the anti-egg-serum P-4. Moreover, the number of affected eggs among the responding females was much higher in J-2 than in P-4 and the qualitative response was appreciably stronger. No difference of this sort could be found when the activating potencies of these 2 antisera were compared; both gave rise to visible reactions in the eggs of about $^1/_3$ of the females. With only very few exceptions, eggs from the same individuals and, moreover, about the same number of eggs responded to both antisera. The number of activated eggs is expressed in Table VI as the percentage of the total number counted in all responding females. 250 eggs were counted from

TABLE VI

Jelly-precipitating and activating action of anti-egg-serum P-4 and anti-jelly-serum J-2, studied on the eggs from 132 females of *Paracentrotus lividus*. Each female tested individually. 500 eggs, 50 per cent antiserum, total volume of reaction mixture 1 ml. Observation after 120 minutes at 20°C. For further explanation see text.

Antiserum	Number of females tested	Number of females with eggs responding to		Number of activated eggs as percentage of the total number counted in all responding females
		Jppt action	Activating action	
P-4	132	34	39	26
J-2		61	42	22.5

Abbreviation: Jppt action = Jelly-precipitating action.

each female. The great similarity in the activation figures in response to both antisera was not only of an over-all nature. It also became apparent when the different females were studied individually. Table VII gives a more detailed picture of the results of tests made on the eggs from 5 different females. Differences were also found when comparisons were made between different anti-egg-sera.

TABLE VII

Eggs from 5 females of *Paracentrotus lividus*, each tested individually with anti-egg-serum P-4 and anti-jelly-serum J-2.

500 eggs, 50 per cent antiserum, total volume of reaction mixture 1 ml. Observation after 120 minutes at 20°C.

Antiserum	Per cent of eggs visibly affected. Female No. 1		2		3		4		5	
	Jppt	Act	Jppt	Act	Jppt	Act	Jppt	Act	Jppt	Act
P-4	~40	20	0	14	<10	45	0	20	~20	16.5
J-2	~60	18	0	13.5	~30	56	~100	31	~100	15.5

Abbreviations: Jppt = Jelly precipitation
Act = Activation.

Wrinkling and activating capacity of the antisera also seemed to be unrelated. It would not have been unreasonable to expect that differences in wrinkling and activating potency between two antisera were merely due to differences in concentration of the same type of antibody. A low antibody concentration in a serum would then only bring about wrinkling, while a high concentration would lead to activation, that is to say, membrane formation and nuclear activation—provided the eggs were reactive. That this was not the case is illustrated by the data from a typical experiment given in Table VIII. One can see that the antiserum P-1, even at low concentrations, activated a considerable proportion of the eggs but did not cause the slightest sign of wrinkling. With the antiserum P-7 the results were the opposite. Whatever the nature of the differences in antisera, they could hardly be ascribed to differences in antibody concentration only.

The question of a possible distinction between wrinkling and activation demands still one more comment. Wrinkling has been defined as changes in the cortical layer of the eggs which do not lead to membrane formation and nuclear activation. The latter two features may be used to characterize the activating effect of the antisera. The data at the end of Table VIII intimate a simultaneous occurrence of both activities. This claim may find its justification in the appearance of the morphological events: membrane formation invariably occurred within 20–30 minutes and was almost always followed by nuclear activation. Visible wrinkling occurred after 50–60 minutes, in the manner shown in the photographs in Figs. 1 and 2 and independent of whether the affected egg already had formed a membrane or not. On the other hand, the surface of all the eggs in an experiment remained completely smooth if the antiserum concentration was too low or the number of the eggs too high. Cf. the data for 5–20 per cent concentration of antiserum P-1 in Table VIII.

TABLE VIII

Jelly-free eggs from 1 female of *Paracentrotus lividus* tested with antisera P-1 and P-7, diluted with normal serum to a total serum concentration of 80 per cent.

500 eggs and varying concentrations of antiserum, total volume of reaction mixture 1 ml. Observation after 180 minutes at 20°C. For explanation see text.

Concentration of antiserum in per cent	Per cent of eggs responding			
	P-1		P-7	
	Wrinkling	Activation	Wrinkling	Activation
0	0	0	0	0
5	0	12	~20	0
10	0	32	~60	0
20	~5	52.5	~80	~1
(80)	(most eggs, weak)	(60.5)	(most eggs, strong)	(8)

These morphological facts and the high variability of the antiserum action constituted, in addition to the differences described in the preceding sections, the main reasons for distinguishing between the wrinkling and activating action of the antisera. It may be useful as a working hypothesis, though considerably more experimentation is needed to prove its validity.

The evidence obtained from testing 10 different anti-*Paracentrotus*-sera on homologous egg material can be summarized in the following, thus far purely descriptive, statements (cf. Table IX).

1. All antisera except one (P-8) had a jelly-precipitating action when tested on reactive eggs. Two anti-egg-sera (P-6 and P-7) and both anti-jelly-sera (J-1 and J-2) had a strong jelly-precipitating effect. When tested at 50–80 per cent concentration on eggs varying from 500–1000 in number, a maximum number of eggs was affected. The response then could not be increased by decreasing the number of eggs and/or increasing the absolute amount of the antiserum in the reaction mixture. Furthermore, the reactions in these antisera were relatively strong and usually accompanied by a contraction of the jelly layer. The other antisera had a weaker jelly-precipitating activity. When tested under experimental conditions identical with those just mentioned, optimal response was not obtained. These antisera never acted upon egg material which did not respond to exposure to one of the first-mentioned antisera.

2. All antisera exerted a wrinkling activity on almost the entire egg material tested. The most potent antisera were the 2 strongly jelly-precipitating sera P-6 and P-7. When 500–1000 eggs were used for testing, most of them were strongly affected even at 50 per cent antiserum concentration. All the other antisera gave weaker wrinkling effects. Although most of the eggs were affected by such antisera in reaction mixtures containing 1000 eggs and 80 per cent antiserum, the qualitative response was much weaker. Except for the antisera P-6 and P-7, no direct correlation has yet been found between jelly-precipitating and wrinkling activity.

3. All antisera except P-8 activated (membrane formation and nuclear activation) a certain percentage of the egg material. The antisera P-1, P-2 and P-3 had a strong activating effect. When tested under conditions similar to those mentioned above, a maximal number of eggs responded even at moderate antiserum concentrations (cf. Fig. 6). The antisera P-4, P-5 and J-2 had a moderate activating capacity. They activated the same egg material but affected fewer eggs when used under identical experimental conditions as the three strong sera. Improving the experimental conditions sometimes led to an improvement in the response, and titration curves similar to those in Fig. 6 could then be obtained. The antisera P-6, P-7 and J-1 were weak; they still acted on the same egg material as the strong antisera, but only a small percentage of the eggs was visibly affected even at improved experimental conditions. Eggs which were not affected by P-1–P-3 could never be activated by any of the other antisera. Removal of the jelly layer from the eggs usually improved the response to all antisera. Except for the negative correlation between jelly-precipitating and wrinkling on the one hand and activation on the other with the antisera P-6 and P-7, no significant correlation between the activities of the different antisera has as yet been found.

The properties of the antisera mentioned here have been tabulated in Table IX, together with other data of importance in this connection. A study of the data for the anti-egg-sera shows that the activating potency of the

TABLE IX

Mode of preparation and properties of anti-*Paracentrotus*-sera used in this study.

Abbreviations: Jppt = Jelly precipitation, Wr = Wrinkling, Act = Activation.
A–F = Samples taken from different batches of eggs, lyoph = Lyophilized eggs, frozen = frozen eggs
presens = Presensitization, i.v. = Intravenous, adj = Adjuvant, c = Complex pattern, s = Simple pattern.

0 = No visible action, ± = Very weak, + = Weak, + + = Moderate, + + + = Strong action.

Antiserum	Number of rabbits	Antigen used for injection	Method of injection[1]	Amount of antigen injected/rabbit (mg N)	Reactivity in agar test[2]	Reactivity in egg test[2]		
						Jppt	Wr	Act
P-1	5	lyoph, A	presens	80	+ + +, c	+ +	+	+ + +
P-2	1	„ „	„	80	+ + +, c	+ +	+	+ + +
P-3	3	„ B	„	40	+ + +, c	+	+ +	+ + +
P-4	9	frozen, C	„	25	+ +, c	+	+ +	+ +
P-5	1	„ „	„	25	+ +, c	+	+ +	+ +
P-6	3	„ , D	i.v.	10	+ +, c	+ + +	+ + +	+
P-7	3	lyoph, E	„	10	+ +, c	+ + +	+ + +	+
P-8	5	frozen, C	adj	5	+ + +, c	0	±	0
J-1	1	jelly, F[3]	presens	1.5	+ + +, s	+ + +	+ +	+
J-2	3	„ „[4]	adj	0.5	±, s	+ + +	+ +	+ +

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[1] For explanation see "Material and Methods".
[2] For explanation see text.
[3] Am_2SO_4-precipitated, for further explanation see text.
[4] Alcohol-precipitated, for further explanation see text.

antisera obviously is a function of the amount of material injected into the rabbits. For the other activities no direct correlation of this sort is indicated. Whether the special properties of the antisera P-6 and P-7 were due to the amount of antigen injected, its nature or the route of injection is still quite obscure. This is also the case concerning the possible correlation between the precipitin content of the antisera and their *in vivo* activity. When tested with the double diffusion techniques on agar, all the anti-egg-sera on the list gave rise to complex precipitation patterns, similar to those earlier described [24]. This was also the case with antiserum P-8 which exhibited almost no activity when tested on the eggs.

A few remarks should finally be made on the activity, exhibited by the two anti-jelly-sera. The total amount of antigen injected into the rabbits was much smaller in this case than in the case of the anti egg-sera (the nitrogen content of the jelly preparations used was about 3–4 per cent of the dry weight, the nitrogen content of the egg material 10–12 per cent). J-1 was prepared against a jelly preparation, isolated at pH 5.2 and purified by $(NH_4)_2SO_4$ precipitation as described by Vasseur [53]. When tested in the agar precipitin test, both the antigen and the resulting antiserum gave a strong one-line precipitation pattern as previously described. Antiserum J-2 was prepared by injections of a jelly preparation purified by means of repeated precipitations with alcohol at room temperature [53]. Tested as antigen in the agar test, this preparation was completely inactive. The resulting antiserum gave only a very weak one-line response when tested with a good antigen in the agar test. Both antisera still exhibited a strong jelly-precipitating activity in the *in vivo* experiments. This might indicate that the antigen found in the precipitin test is not identical with the antigen responsible for the *in vivo* reaction. Furthermore, both antisera exhibited wrinkling and activating activity. The significance of these findings will be discussed in the following section.

No systematic study of this sort has as yet been initiated on the other sea urchin species studied. Only three antisera (5 rabbits each) were available for each of the three species. All were made from lyophilized material, injected by the method of presensitization. In all cases the antisera prepared by injection of the largest amount of antigen had the most potent activating capacities.

DISCUSSION

The phenomena occurring in the sea urchin egg upon exposure to antiserum are by no means unusual. Unfortunately, a comprehensive review of the wide field of experimentation concerning the action of antisera on cells is lacking. As things now stand, the available facts have to be taken from as divergent fields as, e.g., the research on blood groups and bacteria, the action of host antisera on parasitic organisms in infection or the action of specific antibodies on cells in embryologic development and growth.

Greatly simplifying the picture given in the preceding sections, we may differentiate between three main response reactions: (1) a coagulation or precipitation reaction in the cell envelope, the jelly layer; (2) a lytic reaction, the wrinkling of the cell surface; and (3) a stimulating reaction, parthenogenetic activation, followed by, or combined with, strongly inhibiting reactions—cell division and development of the activated cell are stopped at an early stage. All these kinds of response reactions to antiserum treatment are well known from work on other organisms. Reference shall only be made to the somewhat similar specific coagulation of the cell envelope, e.g. in Pneumococci, the Neufeld reaction [43], to the immunolysis of blood cells, bacteria and protozoa [3], and to the complex of both stimulating and growth-inhibiting effects of antisera in bacteriology [14], in protozoology [14, 42] and in the field of growth and development [5, 57].

An explanation of the response reactions obtained in our case on the basis of a multiplicity of the antigens involved seems well justified. We have no reason to expect the egg surface to be serologically simpler than that of, e.g., bacteria (cf. [23] for further references). In fact, there can be no doubt that the simplest explanation of the experimental facts put forward in this paper is found by ascribing the antiserum activities to the reaction of antibodies of different specificities with correspondingly different antigens. Some of these can probably be assumed to be situated in or near the surface layer of the cell. Their minimum number would be three or possibly only two. Serological cross reactions, interactions between the antigens themselves and between these and other molecules must be expected to contribute to the complexity and variability of the morphological response. Concentrational effects and varying amounts of blocking antibodies might, on the other hand, offer an explanation of the variability in antiserum reactivity.

The response reactions seen in the microscope are the final results of highly complicated molecular events which of course are difficult to interpret. The evidence presented here is by no means sufficient for far-reaching conclusions concerning the number and nature of the antigens involved in the response reactions, and a great deal of further experimentation is needed.

The nature of the reactions leading to the precipitation of the jelly layer is not yet understood. Precipitation of the jelly layer by antiserum and agglutination which sometimes follows, closely resemble the reactions obtained when basic substances as, e.g., clupein [34] or extracts from the gametes themselves [29, p. 264; 46, p. 189] are added to the eggs. There is no reason to believe that the groups reacting in these cases are identical with those participating in the assumed antigen–antibody reactions. The "precipitation" is thus simply an unspecific response to both specific and unspecific reactions. It seems to involve an irreversible dehydration and probably also structural changes in the jelly substance.

The antigens responsible for envelope coagulation in Pneumococci and other microorganisms are of a carbohydrate nature [43]. This may also be true in the present case. From chemical investigations it is well known that species differences occur in the carbohydrate composition of the sea urchin egg jelly [56]. It should also be noted that the antigen responsible for jelly-precipitation is resistant to trypsin in concentrations which dissolve the vitelline layer of the egg, to treatment with acid at a pH as low as pH 3 and to treatment with absolute alcohol at room temperature. Furthermore, it does not seem to be identical with the antigen in jelly solutions which forms a precipitate when tested in the precipitin tests [24]. (The antigen active in the precipitin test is serologically identical with an antigen from the egg homogenate and is believed to be a protein.) On the other hand, the IO_4^- treatment reported did not seem to weaken the response of the jelly layer, though the typical depolymerizing effect of this agent on the layer could be seen [33]. It might well be that IO_4^- used in higher concentrations and under more favorable conditions would destroy the capacity of the jelly antigen to react with antiserum.

Whatever the nature of the antigen, there can hardly be any doubt that the jelly layer is antigenic. The question then arises whether it contains one or more antigenic specificities. From the chemical evidence available thus far [31, p. 47; 46, 53, 54] no conclusions can be drawn about the serological nature of the jelly substance. The fact that it has been proved to be homogenous in the electrophoresis apparatus [38, 45, 47] would not exclude its antigenic inhomogenity [7, 8]. (Concerning the serological complexity of immunopolysaccharides cf. [43]). The evidence presented in this paper seems to point to the fact that at least one antigen, responsible for the precipitation arising upon antiserum treatment, is predominant in the jelly layer. On the other hand, this antigen seems to be independent of the antigen(s) responsible for wrinkling and activation. Yet, injecting rabbits with purified jelly substance leads to the production of antisera evoking even the other response reactions in the eggs, though to a minor extent. Apart from the possibility that we are dealing with cross reactions between antibodies and related antigens, an occurrence of even other antigens in the jelly would hereby be suggested. Whether this (these) would be inherent in the jelly substance, e.g., in its nitrogenous part, or would consist of "contaminating" substances can of course not yet be decided. It needs hardly be emphasized that even very small amounts of contaminating antigens might bring about a considerable antibody production upon injection into rabbits.

Obviously a great excess of antibody molecules is needed in order to cause a visible manifestation of the jelly precipitation. Yet, eggs were always found which did not respond at all to the antiserum treatment and the reactivity of the jelly layer was clearly correlated to the state of maturity of the eggs. Thus far, it is not known whether these unresponsive eggs absorb antibodies or not. However, it seems reasonable to expect that the increased reactivity in the responsive eggs is due to a real increased "antigenicity" of the jelly layer, possibly brought about by an unmasking of antigenic groups during maturation. Certain data, indicating a change in chemical composition of the jelly layer of the sea urchin *Echinocardium cordatum* during the breeding season, have in fact been reported by Vasseur [54]. According to this author, the actual amount of carbohydrate in the jelly seems to increase during the spawning season, possibly accompanied by a decrease in the amount of "bound" H_2O. Similar results have also been obtained on the eggs of *Paracentrotus lividus* [55]. Changes in antigenic reactivity during the course of maturation might thus be directly connected with underlying chemical changes.

The finding of a seasonal variation in jelly reactivity has some important physiological implications. There can be no doubt that the serological reactivity of the jelly layer parallels the fertilizability of the eggs as determined by the fertilization rate method of Hagström [11]. Hagström has found an increase in the average fertilization rate during the course of the spawning season [10]. Identical results have been obtained by the present author in an attempt to determine the correlation between jelly precipitability and fertilization rate in the eggs of *Paracentrotus lividus*. A low average fertilization rate was obtained in March, gradually increasing during April and May and culminating in June. The changes showed a very close correlation to the changes in jelly precipitability, both on a statistical basis and based on measurements on the eggs from individual females. Furthermore, Hagström also reported that cautious removal of the jelly layer usually leads to a significant increase in the fertilization rate [9]. This was also observed by the author, but in addition it was found that the differences in fertilization rate between jelly-free eggs and normal eggs became somewhat less marked as the season proceeded. In

Paracentrotus lividus, during a two-week period in June, the fertilization rate was at its optimum, and usually 95–100 per cent fertilization was obtained within 5 seconds after the addition of the sperm in both normal and jelly free eggs. These eggs also exhibited an optimal jelly precipitability. A detailed account of the findings briefly reported here will be presented later.

We can thus conclude that, in general, the increased disposition of the jelly layer to respond to the addition of specific antibodies seems to be causally related to its increased disposition to take up or, at least let pass, the homologous sperm in fertilization. According to the views of Tyler and collaborators [46] the jelly layer of the sea urchin egg is identical with fertilizin [18], which on the basis of its specific sperm agglutinating potency, is believed to be essential for fertilization [46]. This hypothesis might find some support in the reported results, which imply a direct correlation between "antigenicity" and specific sperm-engulfing capacity of the jelly layer. However, as has been pointed out above, we do not yet know the real cause of the changes in jelly reactivity during maturation and any conclusions concerning a direct molecular relation between the serological reactions reported here and the assumed fertilizin–antifertilizin reaction in fertilization [46] are therefore purely speculative.

Making allowance for a certain though probably moderate role in fertilization of the specificity of the jelly layer (cf. [21], p. 326) would by no means exclude its main function as a protection for the egg from unwanted activating stimuli. The evidence presented by Runnström and collaborators concerning the inhibition of activation and membrane formation by the addition of jelly substance to the eggs [30, 31] and recently by Hagström concerning the fertilization rate in normal and jelly-free eggs [9] seems quite conclusive in this connection. It should also be recalled that the serological precipitability of the jelly layer usually was weakened considerably when the eggs were repeatedly washed or allowed to stand in sea water for 5 hours before the experiment was begun. This washing and aging was usually accompanied by a significant increase in fertilization rate and fertilizability in general (cf. also [12]). Furthermore, the presence of the jelly layer exhibited a strong inhibitive influence on both wrinkling and activation of the eggs when exposed to antiserum. An absorption of antibodies by the jelly layer may have been partially responsible for this inhibition but an impairing action of the jelly substance itself also seemed to be involved.

As in the case of the jelly-precipitating action of the antisera, an excessive number of antibody molecules per egg was necessary for a visible manifestation of the wrinkling response. The excess antiserum needed in order to obtain wrinkling was appreciably smaller than that needed for visible jelly precipitation. The microscopic feature of the response, its slow course and the almost complete smoothening of the eggs when returned to normal sea water after antiserum treatment, suggest that the initial antigen-antibody combination gives rise to secondary changes, probably involving osmotic alterations, dehydration and changes in the rigidity of the cortical layer. These secondary alterations can be assumed to be responsible for the lytic breakdown of the surface which was sometimes, perhaps always, taking place at one or a few points. The fact that no true outflow of egg cytoplasm occurred through these lesions might suggest the occurrence of precipitation reactions in and beneath the egg cortex. The point of the lytic breakdown of the surface does of course not need to be identical with the point of antigen–antibody combination. Nor does the reversibility of the microscopic reactions imply a corresponding reversibility of the initial serological reactions.

The chemical nature of the antigen involved is not known. It is insensitive to acid treatment (pH 3) and does not seem to be attacked by IO_4^- as used in this study. A complete inhibition of the egg response is, however, brought about by treatment with trypsin even in a very low concentration. The surface layer of the sea urchin egg known to be trypsin-sensitive is the vitelline layer [36]. Treatment of the eggs with trypsin is also known to decrease the general fertilizability of the eggs [2, 28, 58] though simultaneously an increase of cross fertilizability has been observed [2, 15, 16, 48]. The existence of superficial, specific sperm receptors in the vitelline layer has therefore been assumed [15, 31, p. 53]. It would be an appealing idea to assume such sperm receptor molecules to be antigenic and hence available for a serological approach. However, the evidence presented here does not permit conclusions of this sort.

The membranes which are formed when the eggs are activated by antiserum are granular and highly refractile and thus not fertilization membranes in the true sense. They are very similar to the membranes found in eggs, activated by the action of basic proteins as, e.g., clupein or lysozyme [29, p. 278]. However, antiserum-activated eggs, pretreated with trypsin, do not form membranes and the vitelline layer can thus be assumed to take an essential part in the formation of antiserum membranes as well as in that of normal fertilization membranes [31, p. 54]. The slow formation of the membranes, the retarded nuclear activation and impaired cell division are probably also due to a specific action of the antiserum, as normal serum is known to improve these cell activities [31, p. 65]. Another expression of this inhibiting action of the antisera, possibly closely related to the first-mentioned phenomena, is the inhibition of the breakdown of the cortical granules. Eggs, fertilized after mild antiserum treatment [26], often form granular fertilization membranes, more or less resembling the antiserum membranes. On the other hand, eggs activated or fertilized in normal serum always elevate completely normal membranes.

The number of activated eggs varies rather closely with the amount of specific antibodies in a reaction mixture. Furthermore, it seems to be independent of variations in concentration of normal serum components. This suggests in fact that activation in our case would be rather "specific", that is to say, rather directly and causally related to the initiating serological reactions. In any case, it does not seem to be due to "non-specific" secondary reactions, brought about by normal serum or sea water components after previous sensitization of the eggs by an antigen–antibody reaction. Moreover, the evidence at hand also seems to point to the fact that the activation-initiating reaction may be independent of other serological reactions which must be assumed to take place in the treated eggs, e.g., the reaction leading to wrinkling and lysis of the cell surface. Expressing this in another way, we may say that activation might be brought about by an antibody attack upon one single type of antigen from a family of more or less different specificities.

As judged from the general feature of the response, the assumed antigen–antibody reactions do not give rise to an impulse spreading over the entire egg surface [40, 41]. As a great excess of antibody molecules is needed, it seems most likely that a large number of antigen–antibody reactions are necessary for the set-up of an activated state in "submicroscopic units" as postulated by Allen [1]. The normal spreading of an impulse from these activated centers would no doubt be inhibited by other antiserum activities.

A reaction between a special antigen and its antibody might undoubtedly constitute a direct causal event of the complicated reactions involved in the process of egg activation. Whether egg activation by the sperm would be started by a similar trigger mechanism, and moreover whether the same receptor molecules would be involved, is of course open to question but seems by no means to be excluded. The initiation of reaction chains by a serological trigger mechanism is known to occur in anaphylaxis and allergy [e.g. 6, 39]. As has been pointed out [35], egg activation offers several analogies to the conditions prevailing in these fields. Activation of proteolytic enzymes is known to play an important role in the set-up of the allergic conditions [49]. As has been shown recently, the mere addition of an antigen is sufficient to bring about activation of proteolytic enzymes in antiserum [49, 50, 52] or in tissue slices from sensitized guinea pigs [51]. The recent findings of Lundblad [20] concerning the activation of a proteolytic enzyme of cortical origin during the time of membrane elevation in the sea urchin egg is of considerable interest in this connection. Furthermore, liberation of heparin is often observed in connection with the allergic response [51]. The surface layer of the sea urchin egg is known to contain mucopolysaccharides [17, 22], similar though not identical to the heparin-like jelly substance. As shown by means of histochemical methods, these substances are set free at the moment of membrane elevation in the fertilized eggs [17]. We thus may find certain analogies between allergic phenomena and egg activation even here, and a serological approach to the problem of egg activation might offer a valuable angle of attack.

Previously published data [26] showed that fertilization can be strongly inhibited by the treatment of the eggs with homologous antiserum at much higher egg/serum ratios than used in this study. No attempt has been made to prove the identity or non-identity of the fertilization-inhibiting antibodies with those evoking the egg response described in this paper. Further studies on these problems are in progress.

SUMMARY

Upon exposure to rabbit antiserum unfertilized sea urchin eggs of the species *Paracentrotus lividus, Psammechinus microtuberculatus, Arbacia lixula* and *Sphaerechinus granularis* were found to respond in the following way:

1. With most of the homologous antisera a visible precipitation of the jelly layer around the egg was obtained, provided the number of eggs in the reaction mixture was not too high and/or the amount of antiserum not too low. The responsible antigen was resistant to the treatment with acid (pH 3) and low concentration of crystalline trypsin and of IO_4^-. Not all the eggs responded to antiserum treatment. It was observed that the precipitability of the jelly layer varied during the course of the spawning season and apparently was a function of the state of maturity of the eggs.

2. With all homologous antisera wrinkling of the egg surface was obtained. For a visible manifestation of the reaction the egg/antiserum ratio in a reaction mixture had to be low. The manifestation of the response was slow and was completely inhibited by a pretreatment of the eggs with crystalline trypsin at low concentration. Pretreatment with acidified sea water (pH 3) or with IO_4^- in a low concentration did not inhibit the reaction. Transfer of antiserum wrinkled eggs to sea water led to an almost complete smoothening of the cell surface. Almost all eggs reacted upon exposure and no correlation

between the reactivity and the state of maturity of the eggs was observed. The presence of the intact jelly layer around the eggs inhibited the response. Oocytes responded in an atypical way to antiserum and usually became cytolyzed.

3. Most of the antisera exerted an activating action (membrane elevation and nuclear activation) upon a certain percentage of the eggs. Activation in the affected eggs only rarely proceeded beyond the initiation of nuclear activation. Inhibition of development seemed to be a specific antiserum effect also. The reactions were completely inhibited when the egg/antiserum ratio in a reaction mixture was too high. The manifestation of this action was faster than that of the wrinkling action and was inhibited by a pretreatment of the eggs with acidified sea water below pH 5. Treatment with crystalline trypsin did not decrease the response though no membranes were elevated from the affected eggs. The presence of the jelly layer around the eggs had an inhibiting action on the response. As in the case of the jelly precipitation a certain, though weaker, correlation between the reactivity of the eggs and their state of maturity was observed. The potency of the antisera to evoke activation was found to be a function of the amount of the sea urchin material injected into the rabbits. No correlation of this sort seemed to be valid for the other antiserum activities reported.

All the antiserum actions described were highly species specific. Exposure of the eggs to normal rabbit serum produced no effect. Furthermore, no normal serum components seemed to participate in the reactions. The significance of the findings is discussed.

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THE ROLE OF MUCOPOLYSACCHARIDES IN THE FERTILIZATION OF THE SEA URCHIN EGG[1, 2]

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For a number of years one of the writers [40, 41, 42] entertained the idea that activation of the egg upon fertilization or upon treatment with parthenogenetic agents might be due to the removal of an inhibitor. It was supposed that this inhibitor was related to heparin and to the substance of the jelly coat. The proposed scheme was designated as "a modified fertilizin theory in which, however, an inhibitory action is assigned to the fertilizin" [42, p. 315]; cf. concerning fertilizin [23, 52].

In the meantime it was proved by the work of Vasseur [55, 56] that the jelly coat surrounding the egg contains as its essential component a mucopolysaccharide esterified with sulphate. The chemical composition of this mucopolysaccharide varies according to the species or at least the genus as demonstrated by Vasseur and Immers [58]. Contributions to the chemistry of the jelly coat were also made by Tyler [53, 54]. The substance of the jelly coat has a rather strong anticoagulating action in the blood system [14].

Inhibition of fertilization

It was demonstrated that the dissolved substance of the jelly coat exerts an inhibitory effect on the formation of the fertilization membrane at normal fertilization [44, 51] as well as at artificial activation [43]. At sufficiently high concentrations of the substance a considerable inhibition of fertilization occurs [59].

A number of other substances were found to act like the jelly coat: heparin [7, 8], human blood group H-substance, chitin disulphate [59], dextran sulphate [4], chondroitine sulphate [3]. Fucoidin from *Fucus vesiculosus* proved to be inactive in its pure form [3, 57], but another substance present in extracts from Fucus has proved to be a potent inhibitor [7, 59]. It has been purified to a certain degree in our laboratory by Vasseur [57]. The substance has been called Fucus fertilization inhibitor pending its chemical identifica-

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[1] Dedicated to Professor J. Seiler on the occasion of his 70th birthday.

[2] Essential parts of this paper were presented at the Symposium on the Physiology of Fertilization, Palermo, April 1955. Certain new results have been added. Another part of the survey given at the Symposium will be published elsewhere [45].

tion. The designation of the substance is abbreviated to FeInh (Fu) according to the principles proposed by our group [13].

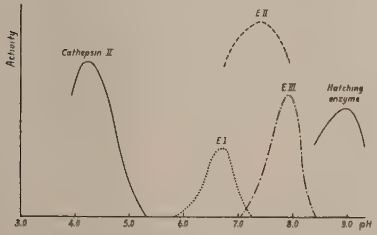
Since the rate of fertilization has been studied more systematically in connection with the inhibition experiments [3, 4, 5], it has been realized that the inhibitors decrease the rate of fertilization rather than cause a block in the absolute sense. Fifty per cent of the control eggs may be fertilized after 15–30 seconds; in the presence of 0.01 FeInh (Fu), 50 per cent of the eggs are fertilized only after hours.

Enzyme activation and inhibition

According to Lundblad [26, 27, 28] at least four different kinds of proteolytic enzymes are present in the eggs, see Fig. 1 [26]. One of these are the kathepsins with a low pH optimum. They exhibit the same activity before and after fertilization. Three other kinds of proteolytic enzymes with pH optima between 6.5 and 8 undergo a strong but temporary activation upon fertilization of the egg. The results obtained by Lundblad indicate that one of these enzymes, designated as *EII* initiates the activation process. Thereafter follows the activation of *EI* and *EIII*, cf. Fig. 2 [26].

The data presented by Lundblad support the view that an activation of enzymes occurs in connection with the activation of the egg. Moreover, Lundblad [26, 27] has shown that FeInh (Fu) and heparin inhibit the activity of the enzymes *EII* and *EIII*. They should thus inhibit or delay a sequence of activations of proteolytic enzymes.

In extended experiments the FeInh (Fu) has proved not only to be an inhibitor of proteolytic enzymes but also of hyaluronidase, ribonuclease,



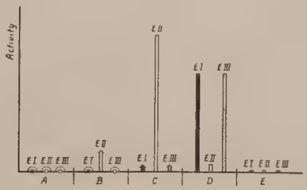


Fig. 1 (left). Diagram showing pH optima of proteolytic enzymes in homogenates of sea urchin eggs. The enzymes *E I* and *E III* proved to be activated by cystein, whereas the activity of enzyme *E II* is lowered by cystein. It has not been possible to determine the pH optimum of enzyme *E II* in detail. The pH optimum of the hatching enzyme is shown for comparison [26, 27].

Fig. 2 (right). A scheme showing the activity of proteolytic enzymes postulated in intact sea urchin eggs before and after fertilization. *A*, before fertilization; *B*, at fertilization; *C*, at membrane elevation; *D*, immediately after membrane elevation; *E*, ten minutes or more after membrane elevation [26, 27].

urease and amylase [3, 57]. The inhibition of hyaluronidase was used as a test in the preparation of the FeInh (Fu). When different fractions were compared, a striking parallelism was found between the inhibitory effects on hyaluronidase and on fertilization. Despite this parallelism no uronic acids seem to be present in the sea urchin egg; cf., also further below.

Even the more active fractions of FeInh (Fu) available are still mixtures of different substances and outward circumstances have delayed further attempts at purification. As shown by experiments on electrophoresis the active part of the preparation is a strongly acid substance which migrates to the positive pole even at pH 3.35 [57]. This acid character seems to be conferred on the substance by esterification with sulphate. The active substance does not dialyze through cellophane and has consequently a macromolecular nature [57].

It is known that heparin is also an inhibitor of a number of enzymes [19]. Probably both FeInh (Fu) and heparin act on enzymes and also on the egg surface by a general reaction with proteins. In favor of this view is the fact that addition of proteins like serum albumin removes the inhibitory effect of FeInh (Fu) and of heparin on hyaluronidase. Likewise serum albumin removes the inhibitory effect on fertilization exerted by both substances [3]. The addition of serum albumin to eggs enhances the rate of fertilization even in the absence of an external inhibitor. This may partly be due to an effect on the sperm. But the presence of serum albumin (concentration, e.g. 0.1 per cent) also enhances the activating action of a hypertonic solution, cf. [60]. The serum albumin probably also counteracts a natural inhibitor residing in the surface of the egg.

Despite our imperfect knowledge about the chemical nature of FeInh (Fu) it has been used extensively in our experiments on fertilization and development [47] because of its high activity even in its impure state. Moreover, it seems that its action is additive to that of a natural inhibitor present in the egg. This point will be further elucidated below.

Effect of sodium periodate

Addition of rather low concentrations of sodium periodate (5×10^{-6}–$5 \times 10^{-5} M$) to the sea water increases considerably the rate of fertilization of sea urchin eggs (*Psammechinus miliaris, Paracentrotus lividus*), cf. [5]. If the concentration of periodate is further increased an activation of the egg occurs. A membrane elevation is observed and the nucleus migrates to the center of the egg. When brought into hypertonic sea water the eggs exhibit the angular form of plasmolysis characteristic of activated eggs, cf. [49].

According to the method of Harvey [9] the eggs were centrifuged on the border between sea water and an isotonic sucrose solution. The rate of elongation of the eggs was measured in the centrifuge microscope [20]. In the presence of periodate ($5 \times 10^{-5} M$) the rate of elongation was 3–4 times greater than that found for control eggs in sea water. Moreover, the rate of elongation increased during an observation for 5–6 minutes (acceleration 4000–5000 g) whereas in the control the rate remained constant during the same period.

The eggs were also centrifuged in a homogenous solution of dextran. As no density gradient is present here, the eggs do not elongate under these conditions, but the stratification of the cytoplasm is more easily observed. No difference in rate of stratification could be observed when control eggs and those subjected to periodate treatment were compared. The difference with respect to elongation rate in the first type of centrifugation experiments must be due to a decrease in the rigidity of the cortical layer of the egg. By similar procedures it turned out that substances which enhance the fertilization rate cause a decrease in rigidity of the egg cortex. FeInh (Fu), on the other hand, caused an increase in rigidity of the egg cortex [20].

Character of natural inhibitor

In one experiment with eggs of *Psammechinus miliaris* the control eggs showed 50 per cent fertilization with a certain sperm suspension after 37 seconds. Upon pretreatment with $2 \times 10^{-6} M$ sodium periodate the 50 per cent fertilization level was reached after 18 seconds. In the presence of $1.8 \times 10^{-2} M$ per cent of FeInh (Fu), 50 per cent of the eggs were fertilized only after 1–2 hours. If, on the other hand, the eggs had been pretreated with $2 \times 10^{-6} M$ sodium periodate 50 per cent of the eggs were fertilized after 120 seconds in the presence of the inhibitor [3, 4]. This means that the pretreatment with periodate caused about a 30–40-fold acceleration of the fertilization rate in eggs exposed to the same concentration of FeInh (Fu). This result is considered to prove that the FeInh (Fu) is additive to a natural inhibitor present in the egg cortex. Owing to the pretreatment with periodate the level of the natural inhibitor decreases. Addition of FeInh (Fu) of a certain concentration now causes a lower degree of inhibition than when the same concentration of the substance was added to non periodate pretreated control eggs in sea water.

The additive effect will probably also be found with other inhibitors mentioned above but so far only FeInh (Fu) has been thoroughly tested in this respect. The action of periodate seems to be rather specific, neither hydrogen peroxide nor potassium ferricyanide nor iodosobenzoate exerted the same improving action on the rate of fertilization as periodate [46]. Periodate like calcium ions exerts an activiting effect on the proteolytic enzyme *EII* [26, 27].

Periodate very quickly depolymerizes the substance of the jelly coat of the sea urchin egg [48]. On the other hand, the action of periodate is not limited to the jelly coat, for eggs deprived of their jelly coat are also influenced by periodate.

In the first place periodate attacks α-glycol groups. Possibly it depolymerizes mucopolysaccharides present in the cortical layer of the egg. The decrease in rigidity of the cortical layer reported above may be the consequence of such a depolymerization. Even if no detailed conclusions can be drawn from the chemical nature of the inhibitors, it is probable that the "natural" inhibitor of the cortical layer has a chemical character which at least in a general way agrees with that of the inhibitors. On this basis the "natural" inhibitor might be conceived of as a mucopolysaccharide with ester sulphate bonds, possibly combined with polyphenol groups.

It seems possible that the natural inhibitor is identical to the jelly coat with respect to its chemical composition. According to Nakano and Ohashi [34] fucose is present in the jelly coat and also in the hyaline layer and in the cortical granules of the eggs of *Hemicentrotus pulcherrimus.* This tends to indicate that the polysaccharides of the jelly coat and those of the egg cortex are identical. However, their results cannot be generalized. A different composition of the polysaccharides of the jelly coat and those of the egg has been demonstrated in our laboratory for three species, *Echinocardium cordatum, Paracentrotus lividus* [15], and *Strongylocentrotus droebachiensis* [17, 18]. The jelly coats of these contain fucose, whereas no fucose was found in the jelly-free eggs.[1] The properties of the carbohydrates present in the eggs have been further studied. From the so-called glycogen fraction [24] of the eggs of *Echinus esculentus* two kinds of mucopolysaccharides were separated. One of these contains glucose as the main and mannose as the minor component. Another fraction also contains glucose as the main but galactose as the minor component. The polysaccharides of these fractions are more firmly bound to the protein than is the case with the real glycogen [17, 18].

Location of mucopolysaccharides

A study of the location of the mucopolysaccharides in the egg was carried out in our laboratory by Monné [29], Monné and Hårde [30, 31] and Monné and Slautterback [32, 33]. These studies have since been continued by Immers. In work on *Paracentrotus lividus* the acetic iron reagent of Hale [6] was extensively used. The sites reacting with the ferri ions stain blue after washing with acetic acid and exposure to potassium ferrocyanide (Prussian blue formation). Acid polysaccharides give a reaction with this method. However, its specificity is limited, cf. [16]. Furthermore, the Hotchkiss method [12] was used, which gives a red staining of compounds containing α-glycol groups.

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[1] The amounts of eggs used for the estimations have been so great that a content of fucose corresponding to the figures of Nakano and Ohashi [34] for the hyaline layer and the cortical granules would have been easily demonstrated.

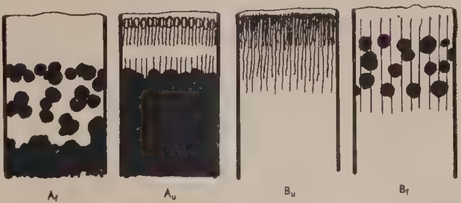

Fig. 3. Diagram of the distribution of polysaccharide material in the cortex of the egg of the sea urchin *Paracentrotus lividus*. *A*, according to Hotchkiss', *B*, according to Hale's reaction. *Au* and *Bu* represent unfertilized eggs, *Af* and *Bf* eggs ca. 5 minutes after fertilization. A comparison of *Bu* and *Bf* shows how a more uniform rim of mucopolysaccharides is converted into more circumscribed particles which eventually are found within the hyaline layer. *Au* shows that the outer rim also stains by Hotchkiss' method. The interior shows a general staining by this method.

In order to study the egg surface and the cortical layer the jelly coat was carefully removed by mechanical means. By virtue of its content of ester sulphate bonds, the jelly is deeply stained by Hale's method.

In the jelly-free eggs an outer zone of the cortical layer stained a pale blue by Hale's method, cf Fig. 3, *Bu*. Appararently this zone is continuous over the whole egg surface. The cortical granules do not seem to be involved in this staining reaction. Like the yolk granules in the interior they stain, however, by Hotchkiss' method. The cortical granules certainly contain polysaccharides, as Monné maintained, but for a final judgement of their character further studies must be awaited.

The ground substance of the endoplasm stains a pale blue with Hale's reagent. This is due to phosphate groups of ribonucleic acid. In certain experiments this was removed by extraction for 15 minutes with 5 per cent trichloroacetic acid at 80°C. The treatment does not affect the staining by Hale's method of the outer rim of the cortical layer. This staining is thus not due to the presence of ribonucleic acid. Lipids which may stain with Hale's reagent are extracted with Carnoy's fluid which served as the fixing agent. The most probable explanation seems to be that the positive reaction of the cortical rim is caused by the presence of mucopolysaccharides with ester bound sulphate. Mucopolysaccharides containing uronic acids have so far not been demonstrated in sea urchin eggs; consequently these compounds may very probably be excluded [18]. The Hale positive rim of the cortex also gives a Hotchkiss reaction, cf. Fig. 3, *Au*. In view of the more general coloration obtained by the method of Hotchkiss the mucopolysaccharide rim of the cortex is not as conspicuous as upon staining by the Hale method. The pictures of Monné and Hårde [31] of centrifuged eggs fixed and stained according to Hotchkiss also demonstrate rather clearly that there is a cortical rim which stains by this method.

The interesting studies of Numanoi [35, 36, 37] should be mentioned in this connection. He found that a sulphatase is present both in the spermatozoon and in the egg. This sulphatase is able to attack the jelly coat. Moreover, sulphatases from different sources are able to bring eggs deprived of their jelly coats to membrane formation and cleavage. Heparin inhibits the effect of the sulphatase.

Changes of mucopolysaccharides at fertilization

The Hale positive rim has the strategical location of a cortical inhibitor. The changes occurring upon fertilization are highly significant with respect to this point of view. During the course of the first five minutes after fertilization the Hale positive rim is broken up into a number of granules which eventually are found in the hyaline layer surrounding the egg, cf. Fig. 3, *Bu*, *Bf*. At the same time the staining of the Hale positive substance changes from pale to deeper blue. The break-up of inhibitory mucopolysaccharides which was postulated by one of the writers seems to be visualized in the process just described. The change of coloration from pale to deeper blue strongly indicates an increased exposure of sulphate groups. Before fertilization these groups are probably bound to proteins of enzymic nature, e.g., the initiating enzyme in the sequence of proteolytic enzymes demonstrated by Lundblad [26, 27].

The Hale positive granules present in the hyaline layer remain there during embryonic development, although changes occur in their staining intensity in certain stages, cf. [17]. If the eggs are brought into sulphate-free sea water the Hale reaction remains positive until the stage of hatching, but thereafter the staining disappears. This is a strong indication of the presence of esterbound sulphate in the granules of the hyaline layer. It is known [10] that lack of sulphate does not affect the development until the free swimming blastula stage. Conversely, after this stage lack of sulphate has a profound influence on development. During the early stages the sulphate may be more strongly bound whereas in later embryonic development a more rapid turnover of sulphate may take place.

Acid formation

The observations reported may also throw light on the longstanding problem of the acid formation which occurs in connection with fertilization of the egg [1, 22, 39]. The last observations concerning this process were carried out by Borei [2] on eggs of *Lytechinus pictus*, apparently a favorable material for such studies. Borei found that the acid formation starts immediately upon fertilization, which was brought about by mixing of eggs and sperm within the divers of the apparatus of Linderström-Lang [25] and Holter [11]. The maximum acid formation is reached within the first minutes after fertilization. Thereafter it subsides. The increase in respiration is much more gradual and starts only when the acid formation has already regressed considerably [2]. The evidence points to the conclusion that the acid formation is closely connected with the cortical changes. If ester-bound sulphate is exposed during the course of these changes an acid formation must occur which should be measurable by manometric methods. There may be an additional acid formation by splitting off sulphate from the mucopolysaccharide of the jelly and the cortical layer by means of the sulphatase of Numanoi [35, 36]. It should of course be desirable to get a more quantitative comparison between break-up of acid mucopolysaccharides and the acid formation.

Further discussion of structural changes

The break-up of the acid mucopolysaccharides would correspond to the impulse which spreads over the egg surface starting from the point of attachment of the spermatozoon. The mechanism of the spreading of the impulse has been discussed elsewhere by one of the writers, cf. [45], where references concerning the subject are found.

After fertilization the hyaline layer is provided with a great number of "granules" of pronounced acid character. Even if the granular aspect may be an artefact caused by fixation etc., it remains obvious that the hyaline layer has components with the character of ion exchangers. These components may be of importance for the ion uptake during the development of the egg and embryo.

The distribution of the acid mucopolysaccharides may also play a role in the type of plasmolysis exhibited by the egg in different stages. When transferred to a hypertonic medium the anisodiametric ripe unfertilized eggs show a fine wrinkling of the surface [50]. After a variable period of time a breakdown of the rim of acid mucopolysaccharide occurs. On the surface of the now spherical eggs a number of goblets are sometimes visible, which contain a mucus-like substance. After fertilization the eggs also change from a more anisodiametric to a spherical form and when immersed in a hypertonic medium they shrink immediately with a smooth surface. During the course of the first 5–10 minutes after fertilization the cortical cytoplasm becomes more rigid; see for references [21]. In the hypertonicity test the eggs now exhibit the angular type of plasmolysis. The discontinuous nature of the mucopolysaccharides of the hyaline layer probably favors the appearance of this type of plasmolysis.

It now seems well established that the activation impulse primarily spreads in the cortical layer of the egg; cf. for references [45]. Secondarily the structural changes spread to the interior of the egg. After fertilization the interior structure becomes more homogenous as is observed both on living and fixed material [38]. The changes are particularly striking when observed on living material with darkfield illumination [38]. The junior author [18] observed pronounced differences between fertilized and unfertilized eggs fixed in Carnoy's fluid and stained with the Hotchkiss method. The red colour is present over the whole egg but there is a striking difference between the unfertilized and the fertilized eggs. In the first place one observes how the stained material forms a coarser more inhomogenous texture in the former, while the latter show a more homogenous distribution of this material. The similarity to the observations on living material is striking despite the fact that Carnoy's fluid hardly should be assumed to preserve the living texture well. Moreover, the red colour is much stronger after staining by Hotchkiss' method in the fertilized than in the unfertilized eggs. This indicates that more α-glycol groups have been exposed. This again bears witness to splitting processes. Also in the interior, linkages between inhibitory carbohydrates and proteins may be broken. This may be the reason for the increased "reactivity" of the cytoplasm, specially of its proteins to which the senior writer has directed attention [40]. Vital staining with methylene blue or neutral red shows that the cytoplasm of the fertilized eggs adsorbs these stains more firmly than that of the unfertilized eggs, cf. [38].

SUMMARY

This paper gives a survey of a number of facts which point to the conclusion that the cortical layer of the unfertilized sea urchin egg contains acid mucopolysaccharides which act as enzyme inhibitors. The natural or artificial activation of the eggs probably causes a break-up of the inhibitory substances and thus releases certain enzyme systems. The results of staining the eggs by the methods of Hotckhiss and Hale seem to bear out these conclusions.

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PHYSIOLOGICAL ANALYSIS OF THE CORTICAL RESPONSE OF THE SEA URCHIN EGG[1]

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The importance of the cortical change in the egg during the process of fertilization has been recognized since Loeb's earlier work fifty years ago and there have been numerous other works on that problem. One of the important findings was on the cortical granules of the sea urchin egg. In 1939, Moser [5, 6] succeeded in observing their changes at the time of fertilization and activation. Since his work, considerable progress has been made in our understanding of the mechanism of the membrane formation. Motomura [7], for instance, demonstrated that some portion of the cortical granules after their breakdown forms the precipitation membrane on the inner surface of the elevating vitelline membrane. Runnström and others [14, 15] observed the behavior of the cortical granules and their derivatives after activation under certain conditions, and presented an interesting conclusion. Endo [2] also made further researches on their behavior, using Japanese sea urchins. Monné and Hårde [4] reported on microchemical properties and physiological role of the cortical granules. In recent years, Rothschild [8, 9, 10, 11, 12, 13] studied the cortical response from the view-point of the mechanism of monospermy, and contributed many remarkable results. These contributions are so well-known that I do not need to describe them in detail.

In 1953, this author published two reports [16, 17] on some experiments which had been done in the hope of analyzing the cortical response physiologically. More recently, Allen [1] also succeeded in his experiments in analyzing the cortical response and reported several interesting results.

There are, I believe, several important problems as to the cortical change at the time of fertilization. One is why does a spermatozoon induce the cortical change not only at the point of its attachment, but also on the entire surface of the egg. It is a well-known fact that the cortical granules begin to break down at the point of sperm-entry and in a few seconds their breakdown spreads all over the egg surface. The mechanism of this phenomenon has, up to the present time, been subject to relatively little investigation, although some hypothetical opinions have occasionally been presented. For instance, it has been suggested repeatedly by several workers that some sort of invisible change is complete before any visible change occurs at the time of fertilization. It might be postulated that the breakdown of the cortical granules follows such an invisible change, but as a matter of fact there has been no evidence to prove causality by an invisible change.

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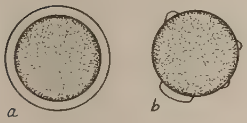
[1] Presented before the Symposium on the Physiology of Fertilization, Palermo, April 26–30, 1955.



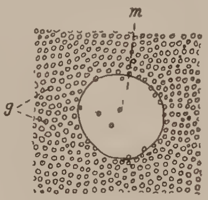


Fig. 1 (left). Eggs treated with sodium choleinate. *a*, an egg with completely elevated membrane; *b*, an egg with partially elevated membrane.
Fig. 2 (right). The cortex of the egg with partially elevated membrane. The cortical granules are broken down only in the area where the membrane is elevated. *g*, cortical granules; *m*, partially elevated membrane.

I have been studying the problem of the cortical response in the sea urchin egg and the results obtained enable me to discuss its physiological nature to some extent. A brief summary of my earlier experiments already published will be given here and some of my later work will subsequently be reported.

Cortical Response to Sodium Choleinate and Wasp-venom

Experiments with sodium choleinate.—The sea urchin employed for these experiments was *Hemicentrotus pulcherrimus* which was previously classified as of the genus *Strongylocentrotus*. Its egg is relatively transparent of an orange-yellow colour and the cortical granules are clearly visible in the living state without centrifuging.

Sodium choleinate is an effective reagent to induce the formation of the fertilization membrane. When unfertilized eggs are put into the solution of sodium choleinate at the optimum concentration, formation of the fertilization membrane normal in appearance develops within a few minutes. However, the mode of membrane formation is rather different from that which takes place when the eggs are treated with butyric acid or urea solution. When sodium choleinate is used, the whole surface of the egg does not become rough before membrane elevation. The membrane begins to rise in one or more places, and a few minutes are required to complete its full elevation (Fig. 1).

If eggs are washed with normal sea water when elevation of the membrane has just taken place only locally and not over the entire egg surface, the membrane formation is interrupted. The cortical granules have been broken down only in that part of the egg cortex where the membrane is separated remaining intact in the other areas (Fig. 2).

In normal fertilization the breakdown of the cortical granules begins at the point of sperm-entry and then spreads towards the opposite side of the egg in wave-like fashion. There may be two possibilities to account for the wave-like breakdown of the granules at the time of fertilization. The one is that the breakdown of a cortical granule may be the cause of the breakdown of its neighboring granules. Thus their wave-like breakdown may be of a self-propagating nature. The other possibility is that there may occur an antecedent propagating change in the cortex which may cause the granular breakdown, which appears to be wave-like.

If the breakdown of a cortical granule were to induce the breakdown of its neighbors, then the granular breakdown and the membrane formation would not be interrupted by washing the egg with sea water as in the above-mentioned experiment. The result of that experiment, therefore, constitutes the evidence for the conclusion that the breakdown of the cortical granules at any point is not the cause of the breakdown of the neighboring granules. It seems quite possible, therefore, that some invisible cortical change of a propagating nature must occur for the cortical granules to be broken down at the time of fertilization.

On the other hand the works of Yamamoto [18, 19, 20, 21, 22] on the fish egg, have demonstrated that the first visible change at fertilization is the wave-like breakdown of alveoli which are embedded in the cortical protoplasm of ripe unfertilized eggs. He postulated that a reaction of some kind may be provoked by the sperm and conducted in a wave-like fashion, causing the alveolar breakdown, and he termed this invisible wave the ''wave of fertilization'' or ''fertilization-wave''. The invisible cortical change in the sea urchin egg resembles the fertilization-wave in the fish egg in its propagating nature, in its occurrence prior to the breakdown of the cortical structure, and in its function of causing the breakdown of the cortical structure. Therefore, I wish to call the invisible cortical change in the sea urchin egg the ''fertilization-wave'', using his term.

A result similar to that of sodium choleinate is obtained with wasp-venom.

Partial Exposure of the Egg Surface to the Activating Reagent

The cortical response to an insufficient treatment with sodium choleinate or wasp-venom is somewhat different from that to an insufficient treatment with butyric acid or urea solution. By means of either of the former, a partial separation of the membrane can be induced, while either of the two latter seldom induce such a strictly partial membrane separation. The question arises: How can we account for this difference? The possibility may be considered that some stimulating reagents provoke the fertilization-wave which leads to the breakdown of the cortical granules, while other reagents act directly to cause the breakdown of the granules without inducing any propagating response.

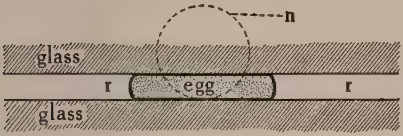


Fig. 3. Diagrammatic view of the compressed egg. *n*, outline of a normal egg; *r*, reagent to stimulate an egg.

In order to investigate whether the effect of a stimulating reagent is of a propagating nature, the egg surface was partially exposed to the activating reagent and the response in the unexposed surface was examined. When the cortical granules in the exposed part are broken down and those in the unexposed part remain intact, it may be considered that the effect of the reagent is not of a propagating nature. But when the cortical granules on the whole surface, including the unexposed part, are broken down, the effect of the reagent may be considered of a propagating nature.

Eggs deprived of their jelly-coat are placed on a slide and covered with a cover-glass. Bearing down slightly on the cover-glass the eggs are flattened. Thus, the upper and lower parts of the eggs adhere closely to the glass-surface, while the equatorial region of the egg is in contact with the sea water (Fig. 3). Several drops of the reagent to be tested are placed on the slide at one end of the cover-glass and a small piece of filter paper is applied at the opposite end. After an appropriate time, the reagent is replaced by sea water. Thus the equatorial region of the egg is exposed to the reagent, while the upper and lower parts are not influenced directly by the reagent.

When sodium choleinate or wasp-venom is used, the cortical granules in the equatorial region soon begin to break down, but in the upper and lower parts the granules remain completely intact. Monogen, a detergent common in Japan, has the same effect. These results suggest that the cortical change caused by treatment with sodium choleinate, wasp-venom and Monogen is not of a propagating nature.

On the other hand, when the egg surface is partially exposed to butyric acid or urea solution, the cortical granules in the equatorial region of the egg begin to break down and within a few seconds their breakdown rapidly proceeds in the upper and lower unexposed part of the egg. It is concluded that the effects of these reagents are of a propagating nature.

It has been found that distilled water is also an activating agent, having the same effect as that of butyric acid.

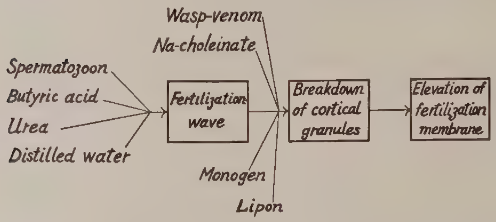


Fig. 4. Diagram showing the process of the cortical change.

The above-mentioned results make it possible to classify the stimulating reagents in two groups according to the nature of their effects. This means that the effects are not always the same as far as the invisible cortical response is concerned. The first group includes reagents such as butyric acid, distilled water and urea solution. The cortical change provoked by these reagents is of a propagating nature and there must be an invisible wave-like change underlying the breakdown of the cortical granules. It may be suggested that this change would be essentially the same as that resulting from the entrance of spermatozoa. If this be true, then it may be said that the reagents of the first group induce the fertilization-wave.

To the second group belong reagents such as wasp-venom, sodium choleinate and Monogen. Their response is of a non-propagating nature. It is concluded that reagents of the second group induce the granular breakdown without intervention of the fertilization-wave.

Fig. 4 gives us the scheme of the cortical change at fertilization and activation, hypothetical in parts. It shows that the spermatozoon and the reagents of the first group give to the fertilization-wave and this causes the breakdown of the cortical granules. The reagents of the second group cause the breakdown of the granules without intervention of the fertilization-wave. The granular breakdown, then, is followed by the elevation of the fertilization membrane, regardless of the nature of the activating reagent.

In 1954, Allen [1] reported several important results from his experiments on the cortical change and the fertilization impulse. He employed the capillary method for partial surface exposure. According to his findings the types of response observed can be divided into three groups and the fertilization impulse or fertilization-wave is more specific than assumed by me.

Experiments with Narcotized Eggs

In the next series of experiments, the influence of narcosis on the cortical change was studied. We may expect that the invisible cortical change of a propagating nature, the fertilization-wave, might be influenced by the change of irritability of the egg cortex.

Unfertilized eggs of *Hemicentrotus* were narcotized by means of a 6 per cent solution of urethane in sea water, and after washing in normal sea water, they were fertilized or artificially activated. Since the urethane solution was considerably hypertonic, control experiments were run with a hypertonic sea water containing excess NaCl, and it was found that the hypertonicity of this degree did not influence the experimental results.

It was found at first that when the unfertilized egg is completely narcotized, no cortical change is provoked at fertilization (Table I). However, inhibition of the response by urethane is reversible (Table II). For instance, when

TABLE I

Cortical change in narcotized eggs.
(Narcotized with 6 per cent urethane sea water) (9° C).

Time of immersion in urethane sea water (seconds)		0	10	20	30
Percentage of eggs which showed cortical change	after insemination	99	98	81	0
	after urea treatment (7 s)	98	99	80	0
	after butyric acid treatment (1 m)	99	98	76	0

TABLE II

Reversibility of narcosis.
(Narcotized with 6 per cent urethane sea water) (9° C).

Treatment	Immersed in urethane sea water for 30 seconds	Immersed in urethane sea water for 30 seconds and then kept in normal sea water for 12 minutes
Percentage of eggs which showed cortical change after insemination	0 per cent	98 per cent

unfertilized eggs are immersed in urethane sea water for 30 seconds and are inseminated immediately after their return to normal sea water, no cortical change is shown, although spermatozoa enter the eggs. But when the eggs are kept in normal sea water for 12 minutes before insemination, almost all the eggs recover the irritability. Similarly, butyric acid and urea solution do not provoke the cortical change in completely narcotized eggs (Table I), but the irritability of the egg to these reagents is readily recoverable (Table III). For instance, narcotized eggs immersed in urethane sea water for 30 seconds show no response to urea solution, but after 12 minutes' immersion in normal sea water they recover the irritability. It is also a noticeable fact that the egg once treated with urea solution in narcotized condition, can show the cortical change by a second treatment, after 15 minutes' washing in normal sea water.

TABLE III

Effect of urea on narcotized eggs (9° C).

Treatment	Immersed in urethane sea water for 30 seconds and then treated with 1 mol urea for 7 seconds	Immersed in urethane sea water for 30 seconds and kept in normal sea water for 12 minutes and then treated with 1 mol urea for 7 seconds
Percentage of eggs which showed cortical change	0 per cent	84 per cent

On the other hand, sodium choleinate, wasp-venom and Monogen can induce the breakdown of the cortical granules and the membrane formation in the completely narcotized egg. Sometimes the granular breakdown occurs more quickly in narcotized than in normal eggs. These results indicate that the narcotized unfertilized egg does not respond to the spermatozoa or the reagents which belong to the first group, while the granular breakdown and membrane formation can be provoked by the reagents of the second group (Table IV).

It may be postulated that the cortex of the unfertilized egg is an irritable system and when it is stimulated by a spermatozoon or an activating reagent of the first group, the impulse is provoked and then conducted over the entire cortex. This is the spreading of the fertilization-wave. The narcotic may decrease the irritability of the egg cortex so that the impulse is inhibited and the fertilization-wave does not take place. The reagents belonging to the second group are thought to induce the granular breakdown without intervention of the fertilization-wave. This idea agrees with the fact that the granular breakdown is provoked by sodium choleinate, wasp-venom and Monogen, even in the narcotized egg.

TABLE IV

Effects of various reagents on narcotized eggs.

Reagent	Cortical change in narcotized eggs
Butyric acid	–
Urea	–
Formalin	–
Sodium choleinate	+
Monogen	+
Wasp-venom	+
Saponin.	+

I will take this opportunity to report on some experiments with other activating reagents. Formalin was found to be a reagent effective in inducing membrane formation in eggs of *Pseudocentrotus depressus* (Table V). Stock solution (1.4 cc) of formalin (about 40 per cent) was added to 100 cc of sea water. Unfertilized eggs were put into this solution and after 10–30 seconds they were washed with normal sea water. The cortical change occurred and the fertilization membrane was formed. Experiments with narcotized eggs were performed with formalin sea water and it was found that the response to formalin was inhibited in narcotized eggs.

TABLE V

Activation with formalin (*Pseudocentrotus*, 17° C).

Time of immersion in 1.4 per cent formalin (seconds) .	5	10	15	20	25	30
Percentage of eggs which showed membrane formation	0	98	100	99	71	12

Saponin is a well-known reagent for activation since Loeb's work. Its effect on narcotized eggs was also examined. In this case no inhibition was observed.

Summation of Insufficient Stimuli

Generally speaking, the success of artificial activation of the egg depends upon the irritability of the egg, the concentration of the activating reagent and the time-length of exposure to the reagent. This relation is especially clear cut when butyric acid or urea solution is used. For example, eggs treated with 1 mol urea for 5 seconds show no visible change, while almost all those treated for 7 seconds show full membrane elevation. It may be interesting, by summing up repeated insufficient stimulations, to study if activation occurs or not. According to Lillie [3], starfish eggs can be activated by summing up insufficient stimuli of heat and butyric acid. I am carrying out some experiments on this problem using sea urchin eggs.

Experiments with butyric acid.—Eggs of *Paracentrotus* can be activated by 30 seconds' treatment with 6 per cent butyric acid (6 cc of 1/10*N* butyric acid + 94 cc of sea water) at 16° C, but cannot be activated by 20 seconds' treatment using the same solution. Experiments were performed to study the effect of summation of such insufficient stimuli. The eggs which had been treated with butyric acid for 20 seconds were washed with and kept in sea water for 20 seconds. They were then treated again with butyric acid for 20 seconds. It was found that repetition of these insufficient stimuli could well provoke the activation (Table VI).

TABLE VI

Summation of insufficient stimuli of butyric acid (*Paracentrotus*, 16° C).

Time of immersion in 6 per cent butyric acid * (seconds)	10	20	30	40
Percentage of membrane formation	0	0	93	99

Eggs treated with 6 per cent butyric acid for 20 seconds are washed with sea water and after 20 seconds are again treated with butyric acid.

Time of the second immersion in 6 per cent butyric acid* (seconds)	5	10	20	30
Percentage of membrane formation	0	62	97	98

* 6 per cent butyric acid: 6 cc 1/10*N* butyric acid plus 94 cc sea water.

However, it was also found that when the interval between the two stimuli was more than 3 minutes, their summation was ineffective. In the second treatment after 3 minutes' interval, 40 seconds were needed for successful activation. This indicates that the effect of the first insufficient stimulus was lost during the interval of 3 minutes, and that the irritability of the egg decreased considerably. It may be safely said that summation of two insufficient stimuli can well provoke the activation if the interval between the stimuli is sufficiently short.

Experiments with butyric acid and formalin.—It was found that *Pseudo-*

centrotus eggs treated insufficiently with butyric acid can be activated by an insufficient treatment with formalin (Table VII). Eggs of *Anthocidaris* and *Hemicentrotus* are usually activated with difficulty when formalin is used, but interesting results were obtainable with eggs of those species. When these eggs have once been treated insufficiently with butyric acid, they can be easily activated with formalin. The eggs which have been treated with formalin for some time, can be activated by relatively short treatment with butyric acid (Table VIII). These results suggest that stimulus of formalin is too weak

TABLE VII

Summation of different stimuli (1). (*Pseudocentrotus*, 15° C.)

Membrane formation with formalin.

Time of immersion in 1.4 per cent formalin (seconds)	10	15	20
Percentage of membrane formation	0	47	94

After an insufficient treatment (10 seconds) with butyric acid, eggs are treated with 1.4 per cent formalin.

Time of immersion in 1.4 per cent formalin (seconds)	5	10	15	20
Percentage of membrane formation	0	14	94	93

TABLE VIII

Summation of different stimuli (2). (*Hemicentrotus*, 15° C.)

Membrane formation with formalin.

Time of immersion in formalin s.w. (seconds)	10	20	30	40	60
Percentage of membrane formation	1	1	2	4	2

Membrane formation with butyric acid (6 per cent).

Time of immersion in butyric acid (seconds)	8	10	15	20
Percentage of membrane formation	0	4	90	92

After 8 seconds' treatment with butyric acid, eggs are treated with formalin (1.4 per cent).

Time of immersion in formalin s.w. (seconds)	10	20	30	40
Percentage of membrane formation	0	42	96	45

to provoke activation in the eggs of these species, but the effect of formalin is to complete the stimulation partially made with butyric acid. It is also suggested that the irritability of *Pseudocentrotus* eggs to formalin is higher than in *Anthocidaris* and *Hemicentrotus* eggs.

On the basis of these results, it is concluded that summation of different stimuli using butyric acid and formalin can provoke the impulse. It is possible that a stimulus of any kind using any reagent of the first group, could cause the same impulse. On this point, there has been a question as to whether impulses provoked by various stimuli are of the same or different nature. But I am inclined to think that the same impulse is provoked when a reagent is used so long as it belongs to the first group. I do not necessarily use the words "stimulus" and "impulse" with their meaning in nerve-physiology. However, my assumption agrees with our knowledge of nerve-physiology.

Nature of the Effect of Saponin

As already pointed out saponin can induce the cortical change in the narcotized egg. Its effect is similar to that of Monogen and wasp-venom, and it seems to belong to the second group of reagents. However, it was found that the cortical change occurred in the whole surface, when the surface was partially exposed to saponin, using the cover- and slide-glass method. Therefore, it may be said that the effect of saponin is of a propagating nature and that it belongs to the first group of reagents.

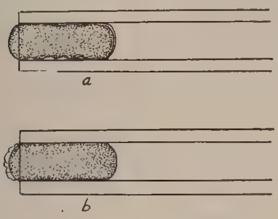
How should we account for this contradiction? My next experiments appear to have given us the answer. The narcotized egg was partially exposed to saponin by the method mentioned above. The result was quite different from that in the normal egg. The cortical change took place only in the exposed equatorial region of the egg and not in the upper and lower regions.

Thus, I consider that saponin not only provokes the propagating impulse but also causes the same effect as Monogen and wasp-venom, that is to say, that saponin shows the same effects as reagents in both the first and second groups.

Experiments of Partial Surface Exposure, Using a Capillary

Allen [1] has performed interesting experiments of partial surface exposure using a fine capillary. I also am carrying out some experiments by a similar method. The study has not yet been completed, but I wish to report some results so far obtained.

An egg is sucked into a capillary, employing a micromanipulator. The egg is elongated considerably because the diameter of the capillary used in these experiments is about 60 microns. The egg is carefully drawn to the end of the capillary, so that a restricted surface of the egg is exposed to an outer medium. When the capillary and the egg is immersed in butyric acid sea water, the cortical change occurs in the unexposed surface, but not in the exposed part (Fig. 5*a*). Such an egg does not change its elongated shape after being blown out of the capillary (Fig. 6*a*). This means that the fertilization membrane has hardened, though incompletely, and that the egg cannot return to its spherical shape.



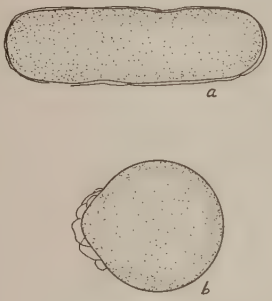


Fig. 5 (left). Diagrams of a partially activated egg in a capillary. *a*, an egg treated with butyric acid; *b*, an egg treated with Monogen.
Fig. 6 (right). Eggs blown out of the capillary after partial activation. *a*, an egg treated with butyric acid; *b*, an egg treated with Monogen.

However, if butyric acid sea water is replaced by normal sea water at an appropriate time, the cortical change occurs in the whole surface. How should we analyze these results? It may be considered that butyric acid can induce the fertilization-wave, and that it inhibits the granular breakdown at the same time. This may be the reason why the granular breakdown occurs in the unexposed part during the time when the capillary is immersed in butyric acid.

When Monogen is used as an activating reagent, both the cortical change and membrane formation take place only in the exposed area (Fig. 5*b*). Such an egg resumes its spherical shape within a few minutes after it has been blown out into normal sea water (Fig. 6*b*). This result is quite different from that with butyric acid and this difference is due to the difference in the nature of the egg surface.

CONCLUSION

This report discusses diverse data on the sea urchin egg. It may be said that the fertilization-wave (fertilization impulse) is the important physiological change of a propagating nature, and that the breakdown of the cortical granules is caused by the fertilization-wave. Many questions may be raised by these findings. What is the physiological and biochemical nature of the fertilization-wave? How does the fertilization-wave cause the breakdown of the cortical structure? What is the relation between the fertilization-wave and the mechanism of monospermy? It is evident that a great deal of work is needed before the cortical change can be analyzed thoroughly. This must be left to future research.

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PHYSICO-CHEMICAL PROPERTIES OF THE FERTILIZINS OF THE SEA URCHIN *ARBACIA PUNCTULATA* AND THE SAND DOLLAR *ECHINARACHNIUS PARMA*[1]

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The fertilizins of eggs of sea urchins and other animals are characterized ordinarily by their ability, when in solution, to cause an agglutination of the homologous sperm. Lillie [11] originally considered that the unfertilized eggs continuously secreted fertilizin as they remained in sea water. However, later experiments by Tyler and Fox [30, 31] and Tyler [20, 21, 22] demonstrated that the source of fertilizin is the gelatinous coat of the egg. The coat slowly dissolves as the unfertilized eggs remain in sea water, giving the false impression of the substance being secreted by the egg. If the gelatinous coat is rapidly dissolved, as by means of sea water acidified to between pH 3 and 5, the amount of fertilizin obtained is as great, or greater, than can be obtained by permitting eggs to "secrete" in sea water for a prolonged period of time. The evidence shows also that there is no macromolecular constituent other than fertilizin constituting the gelatinous coat of the egg.

The view that the gelatinous coat of the egg is the source of fertilizin has been corroborated by experiments of Hartmann [7], Evans *et al.* [4], Runnström and Lindvall [18], Vasseur and Hagström [37], and Monroy *et al.* [15]. It has been reported by Motomura [16, 17] that a "cytofertilizin" can also be extracted in relatively low titer by special methods from eggs that have been deprived of their gelatinous coat. It has been suggested [1] that the eggs had not been completely denuded. Assuming that proper precautions had been taken by Motomura completely to denude the eggs, this obervation would be of interest in connection with such questions as whether the gelatinous coat is originally formed by the follicle cells of the ovary or is a product of the oocyte itself. The presence of a small amount of fertilizin within the egg would tend to favor the former view, which has been adopted by Tyler [23, 25, 27] rather than the latter which Vasseur [35, 36] considers the more likely. It should be noted, in any case, that the growing oocyte can absorb large molecular substances from its surroundings, as illustrated in the experiments of Hevesy and Hahn [8, 9] Chargaff [2], Lorenz *et al.* [12] and others. There is, as yet, no evidence that this may be the case for fertilizin, which is not found in the body fluids or tissues other than the ovary of sea urchins. The finding by Vasseur [35, 36] that ovaries of post-spawning animals yield some fertilizin may well be due to the previous presence of the eggs which have left some gelatinous coat material behind upon spawning or, in many instances, have undergone atresia.

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Solutions of sea urchin fertilizin that are electrophoretically homogeneous and from which practically all nitrogen containing substance is absorbable by homologous sperm are rather easily prepared. Such solutions can be obtained by collecting the eggs in cold sea water, washing with several changes of cold sea water and then extracting with acidified (pH 3 to 5, depending on the species) sea water, precautions being taken to avoid damage to the eggs. Further processing and concentration can be done by dialysis and precipitation with alcohol or ammonium sulphate, as earlier described [25, 27].

Preparations of fertilizin of sea urchins were early found to give both protein and carbohydrate reactions [30, 31, 22, 19] and from later work [23, 24, 25, 10, 33, 34, 36] it has become clear that fertilizin belongs to the class of large molecular substances that may be termed glycoproteins. There are roughly equal amounts of sugar, amino acid and sulphate present in the molecule. In most species that have been examined there are two kinds of sugars and twelve or more different amino acids.

In the reports by Runnström *et al.* [19] and by Vasseur [33, 34, 36] the term "jelly coat substance" rather than fertilizin is employed. However, in view of the above mentioned evidence of identity of the two, it seems reasonable to assume that their analyses apply to what is termed fertilizin. It should also be noted that fertilizins may be readily converted into a non-agglutinating form (termed univalent) by relatively mild treatments and that, in certain species, the fertilizin-sperm system may be ordinarily non-agglutinating but still specifically interacting [21, 22, 23, 13, 14]. It seems reasonable to suppose, then, that in investigations such as those of Runnström *et al.* [19] on the non-agglutinating jelly coat substance of *Psammechinus miliaris*, the analyses apply to what may be termed the fertilizin of the species.

Runnström *et al.* [19] subjected a solution of jelly coat substance of *Psammechinus miliaris* to ultracentrifugation and obtained a main component sedimenting with a sharp boundary and a sedimentation constant of 2.9×10^{-13} along with a small amount of extremely rapidly sedimenting substance. This solution contained 0.26 mg N per ml which, assuming an approximately 5 per cent nitrogen content, would mean that it contained about 0.5 per cent jelly coat substance. Runnström *et al.* mention that the sedimentation constant of the chief component is highly concentration-dependent, a feature that is characteristic of elongate, gel-forming molecules. Tyler [24, 25] reported a sedimentation constant of 6.3×10^{-13} for fertilizin preparations of *Strongylocentrotus purpuratus* which showed only a single boundary in the ultracentrifuge. The solutions were run at concentrations of 0.1 to 0.2 per cent. This higher sedimentation constant might well be due to the determinations having been made on more dilute solutions, as well as to species differences. From the latter value the molecular weight, calculated for spherical shape of the molecules, would be approximately 80,000. However, since the indications are that the molecule is rather elongate the actual molecular weight would be considerably greater. In order to obtain values for the actual molecular weight and shape of the molecule, measurements were therefore made of the diffusion constants along with the sedimentation constants of the fertilizins of *Arbacia* and of *Echinarachnius.* For further characterization of the substance measurements were also made of electrophoretic mobility and viscosity. Brief reports of these values have been previously presented [28, 29].

MATERIAL AND METHODS

Eggs of the sea urchin *Arbacia punctulata* and of the sand-dollar *Echinarachnius parma* were obtained by the method of injection of the animals with isotonic KCl [26]. They were collected in ice-cold filtered sea water and washed a minimum of four times with the cold sea water, at least 20 volumes of the sea water being used per volume of settled eggs for each washing. After the final washing the suspensions were usually concentrated to approximately 10 per cent by volume of settled eggs. The eggs were allowed to settle, the supernatant solution drawn off, centrifuged at 2000 to $3000 \times g$ for about 20 minutes and filtered through rapid filter paper. The solution was then dialyzed against glass-distilled water in the cold. Some samples were precipitated with alcohol but most of the preparations employed were concentrated by evaporation of water from the dialysis bags suspended in front of a fan in a dry room. The bags were suspended on a motor driven "bumper" to reduce deposition of the fertilizin on the dried portions of the cellophane tubing. Samples were then removed for dry weight and Kjeldahl nitrogen determinations and dialysis of the remainder continued against the particular buffer solutions to be employed, precautions being taken to avoid further changes in the concentration of the fertilizin within the dialysis bags.

RESULTS

Sedimentation velocity.—Determinations of sedimentation rate were made in a Spinco analytical ultracentrifuge. Runs were made on three samples of *Arbacia* fertilizin and one of *Echinarachnius.* The *Arbacia* samples were:

(*A*) 0.92 per cent solution in $M/10$ phosphate buffer at pH 6.95, (*B*) 0.46 per cent in $M/10$ phosphate buffer at pH 6.95 (two-fold dilution of (*A*)), and (*C*) 0.21 per cent in $M/10$ borate buffer at pH 9.02. The *Echinarachnius* sample was a 0.39 per cent solution in $M/10$ phosphate buffer at pH 7.02. Photographic records were taken at the time that the refractive boundary was first distinctly visible and at four successive 16 minute intervals thereafter. The first four frames of each of these runs are reproduced in Fig. 1. The displacement of the peak was measured with a metal rule under a magnification of 30-fold, and the sedimentation coefficients were calculated from the equation:

$$s = \frac{\ln x_2/x_1}{\omega^2 (t_2 - t_1)}$$

where x_1 and x_2 are the distances of the peak from the center of rotation at successive times t_1 and t_2 respectively ($t_2 - t_1 = 960$ seconds), and ω is the angular velocity ($\omega^2 = 3.92 \times 10^5$ radians²/sec²). The measurements of displacement of the peak for four successive 16-minute time intervals for each of the four runs gave the following values for the sedimentation coefficients:

Time interval (minutes) .	0–16	16–32	32–48	48–64	Average	
Arbacia fertilizin (*A*) . . .	4.46	4.40	4.33	4.29	4.37	Svedbergs
Arbacia fertilizin (*B*) . . .	5.76	5.77	5.76	5.78	5.77	,,
Arbacia fertilizin (*C*) . . .	6.73	6.73	6.73	6.73	6.73	,,
Echinarachnius fertilizin (*D*)	4.80	4.80	4.77	4.60	4.74	,,

The measurements on the *Arbacia* fertilizin vere not made over a sufficient range of concentration to warrant extrapolation to infinite dilution. They show the marked increase in sedimentation constant with decreasing concentration typical of elongate macromolecules. The higher value obtained at the lower concentration is, of course, colser to what might be obtained by extrapolation to infinite dilution and will be used in subsequent calculations. This may be assumed to be not too far from what would be gotten by extrapolation. The value ($s_{20} = 4.74 \times 10^{-13}$) for the *Echinarachnius* preparation (0.39 per cent solution) is lower than that ($s_{20} = 5.77 \times 10^{-13}$) obtained for a more concentrated (0.46 per cent) solution of *Arbacia* fertilizin. This would indicate that the *Echinarachnius* fertilizin is of smaller molecular size or lower axial ratio or both.

Determinations of the relative viscosities, in an Ostwald viscometer, of the four preparations, *A*, *B*, *C* and *D*, gave the values (cps) 8.22, 2.80, 1.52, and 2.26, respectively.

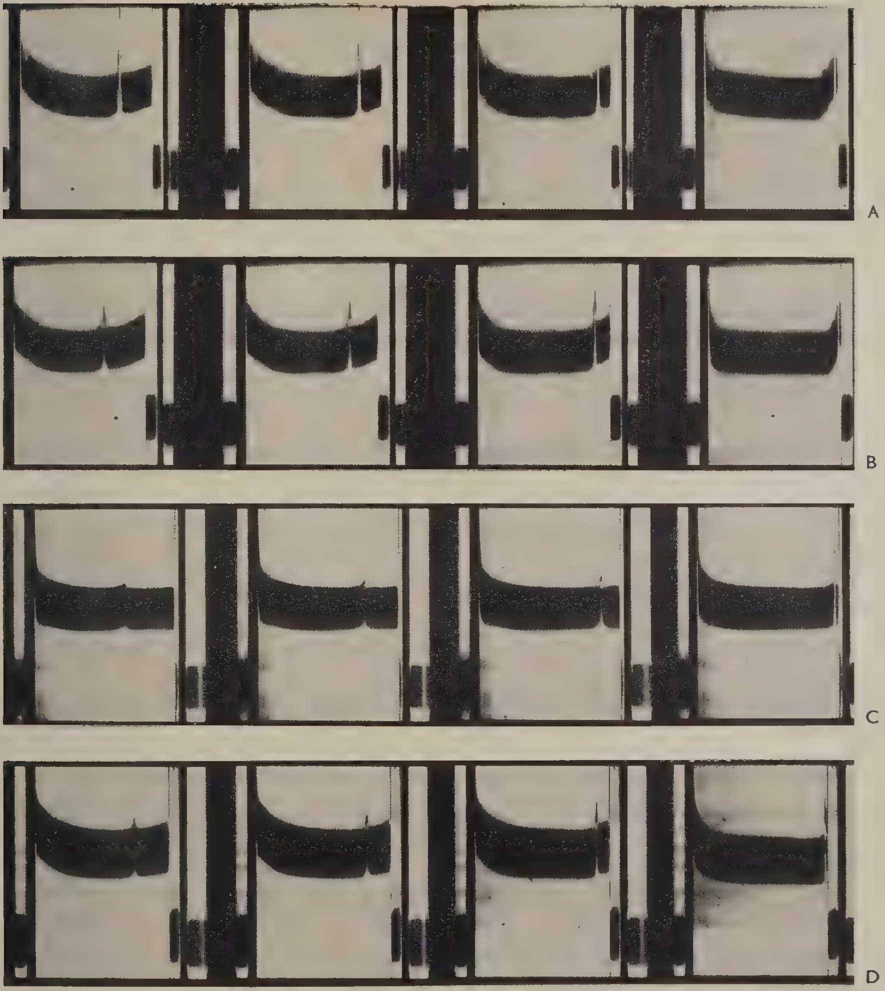

Fig. 1. Photographs of refractive index boundary of fertilizin preparations run in the Spinco analytical ultracentrifuge at 60,000 rpm. The pictures were taken at 16-minute intervals and the sequence is right to left, which is also the direction of sedimentation. Lateral Mag. × 1.48. *A*, *B*, and *C*: *Arbacia* fertilizin at 0.92 per cent in $M/10$ phosphate at pH 6.95, 0.46 per cent in $M/10$ phosphate at pH 6.95, and 0.21 per cent in $M/10$ borate at pH 9.02. *D*: *Echinarachnius* fertilizin at 0.39 per cent in $M/10$ phosphate at pH 7.02.

Partial specific volume.—The apparent partial specific volume was determined by means of a micro-balance and a 1 ml pycnometer. For this purpose a 11.585 mg sample of alcohol precipitated and dried *Arbacia* fertilizin was dissolved in $N/1000$ NaOH. Calculation of the results (see [3], p. 562), gave a value of 0.725.

Diffusion rate.—Measurements were made of the rate of diffusion of the 0.21 per cent solution of *Arbacia* fertilizin in $M/10$ borate buffer at pH 9.02. This was done in a Perkin-Elmer electrophoresis apparatus at 20° C, and over a period of 10 hours. From the measurements of the change in shape of the refractive index boundary, calculations by the maximum ordinate-area method (Greenberg [6] p. 358) gave a value for the diffusion coefficient, D, of 2.16×10^{-7} cm²/sec.

Electrophoretic mobilities.—Determinations of electrophoretic mobilities were made in the Perkin-Elmer design of the Tiselius apparatus. Three runs were made on *Arbacia* fertilizin in 0.2 per cent solutions at different pH and one run was made on 0.2 per cent *Echinarachnius* fertilizin. The following values were obtained:

	cm²/sec/volt
Arbacia fertilizin in $\mu = 0.1$ phosphate at pH 7.02	1.99×10^{-4}
Arbacia fertilizin in $\mu = 0.1$ acetate at pH 4.84	1.57×10^{-4}
Arbacia fertilizin in $\mu = 0.05$ HCl–KCl at pH 2.04	2.02×10^{-4}
Echinarachnius fertilizin in $\mu = 0.1$ phosphate at pH 7.02	1.82×10^{-4}

These values are in the general range reported previously by Runnström *et al.* [19] for the gelatinous coat substance of *Psammechinus miliaris* and by Tyler [25, 27] for the fertilizin of *Strongylocentrotus purpuratus*, and the rapid mobility is indicative of the highly acidic character of the substance. However, the value for the *Arbacia* fertilizin at pH 2 seems anomalous with respect to those at the two higher pH's. This is presumably not due to its having been run at 0.05 molar ionic strength rather than 0.1 molar, since the correction would entail only about 20 per cent reduction of the mobility. It is more likely due to the fact that the fertilizins are unstable below pH 3, as shown by loss of agglutinating activity (though specific reactivity with sperm may be retained). There may then be a splitting-off of some uncharged portions of the molecule at this pH, which would account for the failure of the mobility to decrease regularly with decrease in pH.

Amount of fertilizin bound by sperm.—Two experiments were run to determine the amount of *Arbacia* fertilizin absorbed by homologous sperm. Samples of the fertilizin solutions at 0.2 per cent in sea water were absorbed for one hour at room temperature with equal volumes of serial dilutions of sperm suspension of known sperm concentration. The sperm were removed by centrifugation at about 5000 g for three minutes and the supernatants titrated for sperm-agglutination activity. From the lowest concentration of sperm that just removed detectable fertilizin from the solutions in the two experiments it was calculated that 10^{10} sperm absorb 0.45 to 0.58 mg of this material.

DISCUSSION

Molecular size and shape.—From the values determined above of the sedimentation coefficient, $s_{20^\circ} = 6.73 \times 10^{-13}$, for the 0.2 per cent solution of *Arbacia* fertilizin, of the diffusion coefficient, $D_{20^\circ} = 2.16 \times 10^{-7}$ for a similar solution, of the partial specific volume, $V = 0.725$ and solvent density, $\varrho = 1.007$ a molecular weight of 280,000 is obtained, using the standard equation

$$M = RTs/D\,(1 - V\varrho)$$

in which RT is the product of the gas constant and absolute temperature.

A detailed evaluation of the range of error of the various determinations has not been made. However, it appears that the value for the diffusion coefficient is subject to the greatest error, because of the small size of the refractive index boundaries measured, and it is estimated its value may be in error by as much as 20 per cent. The previously reported [28] figure of 300,000 represents what is considered to be a reasonable approximation when allowance is made for extrapolations to infinite dilution and most likely direction of errors.

From the same data (giving a molecular weight of 280,000 for *Arbacia* fertilizin) a value for the frictional ratio (f/f_0) can be calculated (see [6], p. 392). The value obtained, 2.275, corresponds to an axial ratio of the molecule of 28 to 1 calculated as an unhydrated prolate ellipsoid. Assuming 0.4 gram of water per gram of fertilizin, the frictional ratio corrected for hydration[1] would be 1.96 and the corresponding axial ratio would be 20 to 1.

In the absence of diffusion data for the *Echinarachnius* fertilizin no calculations were made of its molecular dimensions. Assuming that its diffusion constant was similar to that of *Arbacia* and hydration about the same, the relatively lower sedimentation constant indicates smaller molecular size or lower axial ratio or both.

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[1] In the excellent chapter by Lundgren and Ward in *Amino Acids and Proteins* edited by Greenberg [6] there are evidently arithmetical errors in the calculations on the bottom of p. 393 of the factor f/f_e in the Kraemer relation used to correct for effect of hydration. For $r = 0.2$ the value of f/f_e from this equation should be 1.085 instead of 1.01, and for $r = 0.8$ it is 1.28 instead of 1.04. The erroneous figures would indicate little effect of correction for water of hydration on the calculated axial ratios, whereas the effect is evidently quite large.

Number of charged groups per molecule.—From the calculated molecular weight of the *Arbacia* fertilizin and the data on electrophoretic mobility, it is possible to estimate the number of charged groups per molecule, by making use of Stokes' law for spherical particles and adopting a resistance factor to apply to cylindrical particles. Valences estimated in this way show 14.25 effective negatively charged groups per molecule at pH 7 and 11.25 at pH 4.85.

The isoelectric points of fertilizins are evidently very low, as has been previously noted [19, 25]. It seems doubtful that this can be reached without alterations of the molecule induced by low pH. The highly acidic nature of fertilizin is evidently due to the high content of ester-linked sulphate discovered by Vasseur [33].

Number of molecules of fertilizin bound per spermatozoon.—In the experiments described above 10^{10} *Arbacia* spermatozoa were found to combine with an average maximum of 0.5 mg of fertilizin. For fertilizin of 300,000 molecular weight this would be 10^{15} molecules or 10^5 per spermatozoon.

There is evidence [25] that the antifertilizin of the sperm is confined to the region of the head between acrosome and midpiece. This is estimated (as a 1×3 micron cylinder) to have a surface area of 9 square microns. Fertilizin of 300,000 molecular weight and 0.725 partial specific volume would have a molecular volume of 3.6×10^{-19} cm^3. As a prolate ellipsoid of 20 to 1 axial ratio its short axis would be 3.2×10^{-7} cm and its greatest cross-sectional area would be 8×10^{-6} square microns. Attached end on, then, the 10^5 molecules of fertilizin bound by a spermatozoon would effectively cover about 10 per cent of the reactive surface of the head.

It is of some significance that the sperm head is evidently richly provided with receptor groups. The figure of 100,000 such groups represents our first approximation, but it is clear that revisions of molecular weight within the range of error indicated will still give a high figure for the reactive sites on the sperm. It can also be assumed that this is a minimum figure since it seems likely that all reactive sites may not be available for combination with fertilizin, in the absorption experiments. Being so richly provided with reactive sites it now appears more readily understandable that the spermatozoon can effect the rapid union that is customarily observed upon its contact with the egg.

Since, upon fertilization the entering sperm reacts with the fertilizin of the gelatinous coat and surface of the egg it seems likely that the fertilizin thus bound may be brought inside the egg. The question may then be raised as to whether or not fertilizin thus transferred into an internal location may play any role in the activation of the egg. Professor Alberto Monroy and the writer have undertaken some experiments to examine this by means of micro-injection, but certain uncertainties inherent in micro-injection methods (see [32]) have so far frustrated these attempts. It is, however, of interest to note that an analagous proposal has been made [5] for virus penetration of susceptible cells, in that it is considered that the incorporation of a considerable portion of the cell surface material results in the production of complete as opposed to incomplete virus particles.

SUMMARY

Measurements have been made of the sedimentation rates, diffusion, viscosity and electrophoretic mobilities of the fertilizins of the sea urchin *Arbacia punctulata* and the sand dollar *Echinarachnius parma*. From the data for *Arbacia* a molecular weight of 280,000 is calculated, and a value of 300,000 considered more probable. The axial ratio is estimated to be 28 to 1, calculated for an unhydrated prolate ellipsoid and 20 to 1 if 0.4 gram of water is assumed to be bound per gram of fertilizin. The *Echinarachnius* fertilizin sedimented more slowly than that of *Arbacia*. The measurements of electrophoretic mobilities show these fertilizins to be highly acidic as had been found in other species. This data also permitted calculations of valence for *Arbacia* fertilizin as 14.25 effective negatively charged groups per molecule at pH 7 and 11.25 at pH 4.85. Measurements of the amount of fertilizin absorbed show that a single spermatozoon binds approximately 100,000 molecules, and therefore possesses at least that many receptor sites. The relation of this to certain features of fertilization is briefly discussed.

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THE PHYSIOLOGY OF FERTILIZATION IN THE MEDAKA *(ORYZIAS LATIPES)*[1]

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Although extensive studies have been done on the physiology of fertilization in invertebrate ova, only a few works have appeared on the physiological change occurring in fish eggs at the time of fertilization. This is due to lack of appropriate materials as well as to inadequate methods. It has long been known that ripe unfertilized ova of teleosts lose their fertilizability within a short period after they are immersed into water in which fish are living. For instance, ripe unfertilized eggs of the medaka (*Oryzias latipes*), a fresh-water cyprinodont, become incapable of fertilization within a few minutes after they have been immersed into fresh water. For this reason physiological analysis of the fertilization reaction has rarely been performed. Although the "dry method" is excellent for practical use, it is not adequate for the physiological analysis of the fertilization reaction.

Runnström [8] seems to have been the first to observe that ova of a salmonid fish long retain fertilizability in an isotonic salt solution. The fertilization reaction in ova of salmonid fish, however, does not occur in the isotonic solution. The egg of the medaka was found to be isotonic to M 7.5 NaCl [16]. The writer [13, 17] found that unfertilized ova of the medaka retain their fertilization capacity for a number of hours after they have been kept in an isotonic Ringer's solution ($M/7.5$ NaCl 100 parts + $M/7.5$ KCl 2.0 parts + $M/11$ $CaCl_2$ 2.1 parts, pH 7.3 by $NaHCO_3$). Furthermore ova are capable of fertilization and normal development in Ringer's solution.

The female medaka exhibits a maturation cycle of 24 hours and lays a batch of eggs at dawn of every day during the breeding season. In order to obtain ripe unfertilized eggs for research purposes, previously egg-laying females must be isolated from males a day before the experiment is to be performed. It is recommended that females be sacrificed to obtain ripe ova because ordinary stripping method frequently injures ova and causes them to manifest mechanical activation. When ripe ova removed from isolated ovary are immersed into Ringer's solution, only a few ripe ova (5–6 per cent) exhibit auto-activation while the majority remain unchanged. Non-activated ova can be used in fertilization and artificial activation for a number of hours. This property is different from that of ova of *Cyprinidae* and others (except *Salmonidae*) in which 100 per cent of ripe ova manifest auto-activation even in Ringer's solution. The ova of the medaka, therefore, afford us unique material for physiological analysis of the fertilization reaction among teleostean eggs.

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[1] This paper is a contribution to the reports presented in connection with the Symposium on the Physiology of Fertilization, Palermo, April 1955.

Cortical Changes at the Time of Fertilization and Artificial Stimulation

Kagan [3] found in *Fundulus heteroclitus* that the "platelets" embedded in the cortex of unfertilized egg disappear after fertilization. The "platelets" seem to be identical to the cortical alveoli. Tchou and Chen [10] may have been the first to observe the wave-like breakdown of the cortical alveoli at the time of fertilization in the egg of the goldfish (*Carassius auratus*). Independently, the writer [13, 17] observed the same phenomenon in the egg of the medaka. In ripe unfertilized eggs, the cortical alveoli are evenly embedded in the protoplasmic layer except for a small area around the animal pole. The first visible change at fertilization is a wave-like breakdown of the cortical alveoli beginning near the animal pole and ending at the vegetal pole. The change is subsequently followed by the separation of the egg membrane. The formation of the perivitelline space is a consequence of the breakdown of the cortical alveoli. The release of a colloid from the cortical alveoli into the interstice between the plasma membrane and the egg membrane is observable by means of a special method [23]. This is in harmony with previous observations that the egg membrane, which is a dialyzing membrane, is separated by a colloid osmotic pressure [14] and that a slight but significant decrease in the volume of the egg proper takes place after fertilization [15]. Hence it has become clear that the membrane separation is due to a colloid osmotic pressure of the perivitelline fluid which is derived from the content of the cortical alveoli.

Essentially similar cortical response can be induced by a number of stimulating agents. When the cortex of either the animal or vegetal pole is pricked with a glass needle, the tip of which is 15–20μ in diameter, the breakdown of the cortical alveoli begins at the site of stimulation and ends at the antipode. Pricking at the equator also causes the alveolar breakdown at the site of stimulation. The process of the breakdown in this case, however, does not end at the antipode, in a strict sense. The alveolar breakdown near the animal pole proceeds quicker than that near the vegetal pole, with the result that it ends at the point halfway between the antipode and the vegetal pole [17]. The ripe unfertilized ova can be activated thermally by temporary treatment at 45°C [13, 17]. A number of chemical substances also induce the cortical changes. Surface active substances such as sodium taurocholate, sodium glycocholate, sodium oleate, saponin, digitalin and detergents (Aerosol OT, Monogen, Nekal BX and Labolan) are effective activating agents [17, 19]. A short exposure to vapors of lipid solvent such as chloroform, ether, benzene, toluene and iso-amyl alcohol induces almost complete activation while long exposure only results in partial activation [22]. The ripe ova can be activated by electric current. The breakdown of the cortical alveoli and subsequent separation of the egg membrane begin on the anodal and the cathodal sides. The propagation of the change underlying the alveolar breakdown is more rapid near the animal pole than near the vegetal pole [20]. Local stimulation induced by pricking and electric current indicates that there exists an excitation–conduction gradient which is the highest at the animal pole and the lowest at the vegetal pole. The reaction time at the vegetal pole for pricking is about twice that at the animal pole.

By centrifugal force, the cortical alveoli are forced to accumulate in the centrifugal side. There is no relation between the egg axis and the direction of the accumulation. Centrifuged eggs retain their capacity for fertilization and for artificial activation. Even those centrifuged eggs which are alveoli-free in the animal hemisphere can be fertilized. Alveoli-free cortex of centrifuged eggs can be stimulated by pricking. An important fact in fertilization of centrifuged eggs is that only the cortical alveoli embedded in the protoplasm break down while the alveoli which are accumulated in the yolk remain unchanged. This indicates that some sort of propagating change which underlies the alveolar breakdown takes place in the protoplasm either in the presence or the absence of the cortical alveoli, and that the change cannot propagate through the yolk. It has been postulated as the cause of the breakdown of the cortical alveoli that an "impulse" is provoked in the cortical layer by the sperm or activating agents, propagating in a wave-like manner. This impulse was termed the "fertilization-wave" by the writer in 1944 [17, 18].

As an alternative view, an intra-cellular or an intra-cortical diffusion of a sperm substance or of a locally produced cortical substance might be supposed. That such a diffusion concept cannot be applicable is shown by the following heretofore unpublished experiment. When the vegetal pole, which has the lowest sensitivity, is pricked with a 5μ-needle, most eggs show no cortical response. In such cases, not a single alveolus at the vegetal pole breaks down. Such eggs, however, show complete breakdown of the cortical alveoli on stimulation with a $15–20\mu$-needle or on repeated stimulation with a 5μ-needle.

If some cortical substance which causes the breakdown of the cortical alveoli were produced at the site of pricking and diffuse either intra-cellularly or intra-cortically, some alveoli at the site of pricking would break down in response to the pricking with the 5μ-needle even in cases of unsuccessful stimulation. No such condition is realized. Hence the diffusion concept can be excluded.

It may be remarked that the volume of the sperm of the medaka is roughly 1/1,000,000 that of the ovum. It is difficult to suppose a stoichiometrical reaction between an active substance of the sperm and a total of reactive substance in entire cortex of the ovum which would account for cortical changes in fertilization. If a chemical reaction between sperm and egg substances ever takes place, the reaction might be restricted only at the point of sperm attachment. Then cortical changes in the entire cortex proceed autonomously as the consequence of the propagating fertilization-wave in virtue of reactions of substances contained in the ovum itself.

The occurrence of the fertilization-wave at the time of fertilization and activation in sea urchin eggs was confirmed by Sugiyama in 1953 [9] and by Allen in 1954 [2].

The Decrement Conduction of the Fertilization-Wave

The fertilization-wave is defined as an "impulse" which propagates along the cortical protoplasm as the cause of the breakdown of the cortical alveoli. Apparent wave-like breakdown of the cortical alveoli is a consequence of the propagation of this physiological wave. In normal fertilization the breakdown of the cortical alveoli begins at the animal pole and ends at the vegetal pole. In most cases the breakdown is complete. In some cases it is incomplete, i.e., the alveoli at the vegetal pole do not break down. This difference is due to the individuality of the fish from which ova are removed. If the breakdown in an egg is incomplete, all other eggs derived from the same female show the same result. The same batch of eggs which shows an incomplete breakdown in fertilization, manifests complete breakdown when the animal pole is pricked with a needle of 15μ in diameter. This indicates that the intensity of the fertilization-wave provoked by the sperm is weaker than that produced by the 15μ-needle and that it conducts with diminishing intensity. The impulse provoked by the 15μ-needle is so strong that it can become the cause of the breakdown of the alveoli at the vegetal pole although it conducts with decrement during the passage through the cortex. In other words, the all-or-none principle cannot be applicable [18]. This fact is also confirmed in fertilization and artificial activation of incompletely anesthetized eggs. Anesthesia with either *M*/200 phenyl urethane or *M*/200 chloretone in Ringer's solution brings about reversible inhibition in the fertilization reaction. When the eggs are completely anesthetized, no cortical response is provoked by the sperm or by activating agents such as chemical, mechanical and thermal stimuli. In incompletely anesthetized eggs such agents induce partial response, i.e., the cortical alveoli in the animal hemisphere break down while those in the vegetal hemisphere remain unchanged [18, 21].

As has been mentioned before, there exists an excitation–conduction gradient in the unfertilized egg. Since the fertilization-wave propagates with decrement in such a heterobolic system, its velocity in a certain region varies with the direction of the wave. When the animal pole is pricked with a 15–20μ needle, the velocity at the middle region (*AE*) between the animal pole and the equator is 26μ/sec., while that at the middle region (*VE*) between the vegetal pole and the equator is 15μ/sec. (29–33° C). When, however, the vegetal pole is pricked with a needle of the same size, the velocity at *VE* is 22μ/sec. while that at *AE* 19μ/sec. At the equator the conduction velocity is about 20μ/sec. in both directions (unpublished).

It is worth while noting that the decrement conduction of the fertilization-wave (impulse) was also confirmed by Allen [2] in sea urchin eggs.

Chemical Nature of Cortical Alveoli

It is now possible to say that all the teleostean eggs so far examined have the cortical alveoli in unfertilized, non-activated ova [cf. 6]. Their presence was demonstrated also in ova of salmonid fishes in which it has been difficult to observe them because of the opacity of the egg membrane [4, 12].

It is possible to isolate the cortical alveoli from eggs of the medaka by crushing the unfertilized egg with a pair of glass needles in Ringer's solution. This may mean that the fertilization-wave is not instantly established. It may be remarked that the reaction time of the alveolar breakdown is 10–20 seconds. The cortical alveoli isolated in Ringer's solution are relatively stable. The cortical alveolus of the medaka consists of two parts, a thin wall and the interior content. Solubility tests on isolated alveoli indicate that their wall is soluble in such lipoid solvents as chloroform, ether, benzene, toluene and iso-amyl alcohol but insoluble in acetone which is a neutral fat solvent. This indicates that the wall of the cortical alveolus consists of lipoid [21]. That fact was subsequently confirmed by histological method by Aketa [1] in our laboratory. Konopacka [5] seems to be the first to demonstrate histologically that the cortical alveoli (gouttes claires) of the goby and the carp contain a mucoprotein. The polysaccharide nature of the cortical alveoli of some teleosts has been demonstrated by Kusa [7] and Thomopoulos [11]. The same is true of the interior content of the alveoli in the medaka [1].

Chain Reactions in Cortical Changes

It is inferred that the cortical changes involve a series of reactions which are connected in a catenary way. When unfertilized eggs are treated with isotonic oxalate solution, they reversibly lose the capacity for cortical changes on stimulation by pricking. These eggs, however, show the cortical response without further stimulation when transferred to Ringer's solution. In this respect oxalated eggs differ fundamentally from completely anesthetized eggs which also fail to respond to stimulation, but no cortical response takes place on return to Ringer's solution. It requires a further stimulation in order to induce the cortical response. Obviously some event occurs in oxalated eggs when they are pricked in the oxalate solution. To account for the stimulation in oxalated eggs, it is inferred that calcium ions are not necessary in the primary phase but that they are necessary in secondary or later phases of the cortical response. Not only are calcium ions unnecessary in the primary phase but release of Ca ions from the cortical protoplasm at the time of stimulation was demonstrated by a reaction of an exudate of protoplasm with sodium alizarin sulfonate. It was postulated that the first phase (stimulation) of activation takes place even in Ca-free condition, but the second phase (the fertilization-wave) is inhibited in oxalate solution. The cortical changes in *Oryzias* eggs induced by activating agents may be expressed in the following chain reactions:

Stimulants → stimulation → fertilization-wave → breakdown of cortical alveoli → release of alvoelar colloid → membrane separation [24].

For the establishment of stimulation, no calcium ions are necessary. In this phase free Ca ions are released from the cortical protoplasm. Calcium ions may be necessary for the establishment of the fertilization-wave or for the breakdown of the cortical alveoli or for both. As has been pointed out previously [13, 17] calcium ions are necessary in fertilization. In fertilization, union of the sperm with the cortical protoplasm is necessary prior to the establishment of stimulation. Calcium ions seem to be necessary both for the union of gametes and for the later phase or phases in the activation process followed by the establishment of stimulation by the sperm.

Mechanism of Breakdown of Cortical alveoli

As mentioned before, the wall of the cortical alveoli of *Oryzias* egg consists of lipoid, and the isolated alveoli are relatively stable in Ringer's solution.

It was found that their wall is easily dissolved in dilute solutions of venoms from poisonous snakes and bees which contain lecithinase A. When isolation of alveoli is performed with a pair of polished steel needles instead of glass needles, the alveolar wall is dissolved. It was also found that the wall of the cortical alveoli suspended in crushed egg materials is dissolved in the presence of a trace of divalent Fe ions but not of trivalent Fe ions. These experiments suggest that the alveolar breakdown is brought about enzymatically at the time of fertilization and artificial activation. It may be postulated that an esterase is present in inactivated state in the unfertilized egg and the enzyme is activated as a consequence of the fertilization-wave.

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ADDENDUM

On the basis of observation carried out on sea urchin eggs stuck to a glass surface, Runnström and Kriszat (*Exptl. Cell Research* 3, 419 (1952)) postulated that the impulse caused by the sperm attachment is propagated in the cortical layer by a chain reaction. In addition to the work of Allen (1954, *loc. cit.*) this conclusion was also proved beyond doubt for the sea urchin egg by the work of Runnström's colleagues, Allen, and Hagström (*Exptl. Cell Research* 9, 157 (1955)). It is most interesting to find that there is such an agreement among recent works on sea urchin eggs with our results on a teleostean egg.

Experimental Cell Research, **10**, *394–404 (1956)*

ANTIGENS FROM THE YEAST PHASE OF *HISTOPLASMA CAPSULATUM*

I. MORPHOLOGY OF THE CELL AS REVEALED BY THE ELECTRON MICROSCOPE[1]

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Histoplasma capsulatum is conventionally described as an organism surrounded by a capsule [9, 2]. Recently, however, it was reported that the organism was nonencapsulated both in culture media and in animal tissues [2].

Whether or not a microorganism is encapsulated is often difficult to determine under the light microscope. The clear zones which sometimes develop in or adjacent to cell walls during the fixation and staining procedures may be incorrectly characterized as capsular material. These zones may become visible because of a swelling of the walls due to changes in osmotic pressure of the environment, because of retraction of protoplasm from the cell walls, or because of extraction of protoplasmic material diffusing through the cell wall.

A cell wall which is only faintly stained is also revealed by histologic procedures used for demonstration of *H. capsulatum* under a light microscope [1]. It is doubtful whether the existence of a very thin capsule or a slime layer could be demonstrated with such staining procedure.

A more accurate identification of the capsular structure and its differentiation from cell-wall material is obtained with the electron microscope. Such differentiation, however, depends on the use of methods of specimen preparation which do not distort the capsular material. Observations of *H. capsulatum* with the electron microscope have been reported [2] which failed to reveal a

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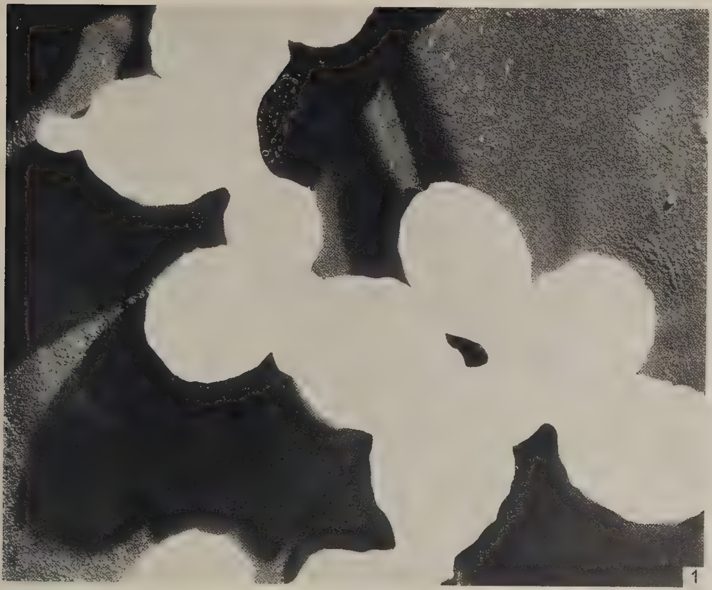
[1] With the technical assistance of W. R. Brown.

Fig. 1. *Histoplasma capsulatum*. Cells adsorbed from the liquid culture medium onto the specimen membrane. No fixation or washing procedures used. Magnification: 21,000 ×.
Fig. 2. *Histoplasma capsulatum*. Cell washed with water prior to specimen preparation. Magnification: 38,000 ×.



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capsule. However, the specimens were prepared by washing unfixed cells with water, a procedure which might remove the capsular material.

Studies of the antigenic behavior of structural elements of the yeast phase of *H. capsulatum* have been in progress [8]. Therefore, it was necessary to determine whether the fungus cell actually possessed a capsule, and whether such a capsule, if present, was immunologically active. In this paper, morphologic studies conducted with the aid of the electron microscope are described not only of *H. capsulatum* but also of *Cryptococcus neoformans,* a yeastlike fungus which has a definite capsule. The true nature of the cell wall and capsule was demonstrated by techniques of specimen preparation wherein uncontrolled distortions of the orginal cell morphology were minimized and wherein controlled distortions were deliberately produced.

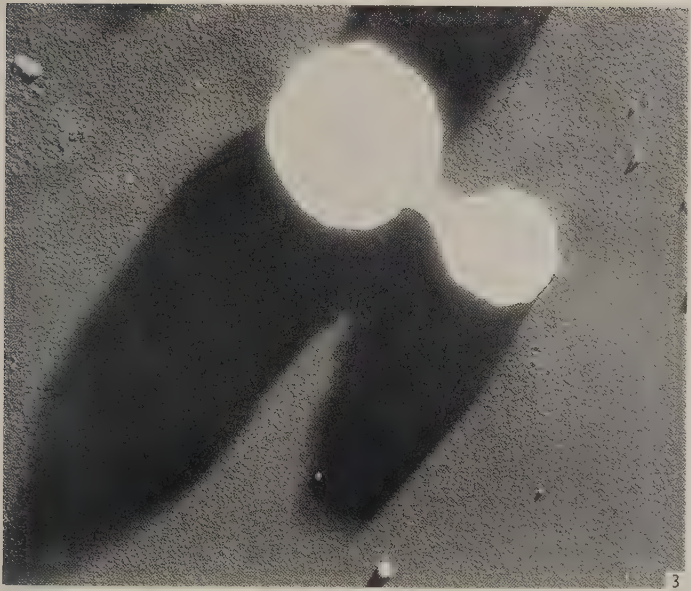
MATERIALS AND METHODS

Fungi. The yeast phase of *H. capsulatum* (isolate No. 6515) [6] was cultured at 37° C on cystine-hemoglobin agar. These cells were then inoculated into a liquid medium containing Difco Casamino acids, cystine hydrochloride, dextrose, and salts, and incubated under continuous rotation at 37° C [7]. *C. neoformans* (isolate No. 3715 obtained in 1947 from a case of meningitis) was cultured in a liquid medium of the same composition under the same conditions. Cultures of *H. capsulatum* were incubated for 72 hours before being harvested, whereas the more rapidly growing *C. neoformans* was incubated for only 48 hours.

Preparation of electron microscopic specimens. –The technique of specimen preparation wherein distortions of the original cell morphology were minimized included direct adsorption of the fungus cells, suspended in culture medium, onto a formvar membrane. A drop of yeast-phase suspension in liquid culture medium was placed on the specimen membrane and then removed immediately with a fine capillary. It was thus possible to obtain areas on the membrane which could be examined under the electron microscope without fixation by chemical agents or exposure to washing procedures (Fig. 1). If air-dried specimens were exposed to vapor of one per cent osmic acid solution for 20 minutes, small residues of culture medium could then be washed off the membrane with distilled water without visible changes occurring in the morphology of the cells (Fig. 3).

Distortions of the original cell morphology were deliberately produced when suspensions of water-washed unfixed cells were prepared onto specimen-supporting membranes and also when unfixed cells were washed with water after being directly adsorbed from the broth onto membranes.

Fig. 3. *Histoplasma capsulatum.* Cells adsorbed from the liquid culture medium onto the specimen membrane, exposed to vapor of osmic acid, and then washed with water. Magnification: 25,000 ×.
Fig. 4. *Cryptococcus neoformans.* Cells adsorbed from the liquid culture medium onto the specimen membrane, exposed to vapor of osmic acid, and then washed with water. Magnification: 17,000 ×.



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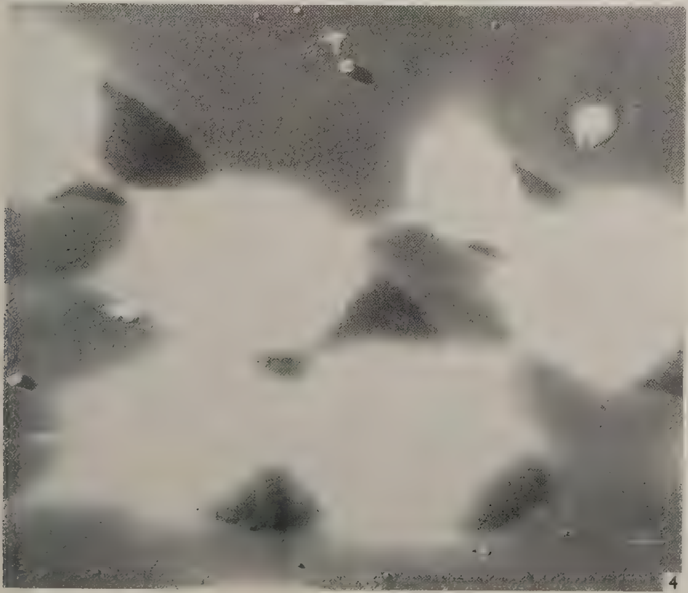

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A platinum-palladium-gold alloy (Usine Genevoise de Degrossissage d'Or, Geneva, Switzerland) was used for shadow casting which yielded a metal film of high electron-scattering power with no perceptible structure when viewed under the electron microscope with a beam of normal intensity. This alloy was readily evaporated from a tungsten wire with a diameter of 0.4 mm. Optimum quality was obtained if the preparations were first desiccated in a large jar until a vacuum of 10^{-5} to 10^{-6} mm of mercury was reached and if this vacuum was maintained during the deposition of the metal film. The shadowing angle was 5 to 1.

RESULTS

Fig. 1 illustrates cells of *H. capsulatum* that were directly adsorbed from the liquid culture medium onto the specimen-supporting membrane. No fixation or washing was carried out. The cells appeared as dense opaque spherical bodies with sharp margins and no obvious capsular material. Characteristic yeastlike budding forms were also seen.

Broad, transparent areas of cell walls became visible when cells of the yeast phase were washed with water by centrifugation prior to deposition onto the specimen-supporting membrane (Fig. 2). The appearance of a peripheral zone of cell-wall material probably resulted from desiccation of the water-swollen cell on the specimen membrane. Similar micrographs were obtained of cells which had been adsorbed from the broth onto the specimen membrane, desiccated, exposed to the electron beam, and then washed with water. Obviously, desiccation in a high vacuum and exposure to electrons did not destroy the swelling properties of the cell wall.

Other cells of *H. capsulatum* were directly adsorbed without washing onto the formvar membrane and then exposed to the vapor of osmic acid. The specimens could then be washed with water without causing visible morphological changes (compare Fig. 3 with Fig. 1).

Cells of *C. neoformans*, which were known to have distinct capsules, were examined with this osmic acid vapor method (Fig. 4). The opaque cells had no sharp margins, as seen in *H. capsulatum* (Fig. 3). The thickness of the capsular material, which was amorphous when viewed under the electron microscope, decreased asymptotically as it spread outward, with no definite boundary discernible. Originally, the capsular material seen in the left part of Fig. 4 appeared without structural differentiation. However, exposure of

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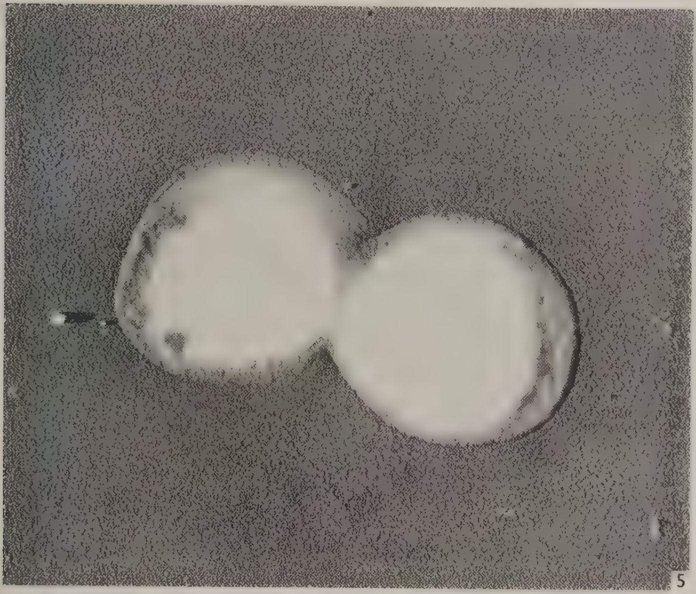
Fig. 5. *Cryptococcus neoformans*. Cells washed with water prior to specimen preparation. Magnification: 21,000 ×.

Fig. 6. *Cryptococcus neoformans*. Cells treated with ethyl-ether while suspended in liquid culture medium and washed with water prior to specimen preparation. Magnification: 14,000 ×.



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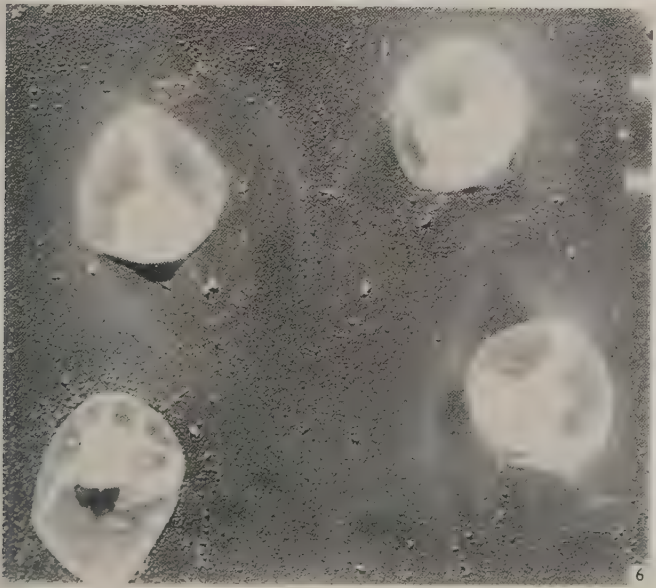

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this area of the specimen to an electron beam of unusually high intensity resulted in formation of visible crystallites in the shadowed metal film. If the capsule were an integral part of the cells seen in Fig. 4, it might be expected that the resulting shadows would be much wider and longer. However, the size of the shadow gave an approximation of the true size of the actual cell body and supported the idea that the capsule was greatly flattened.

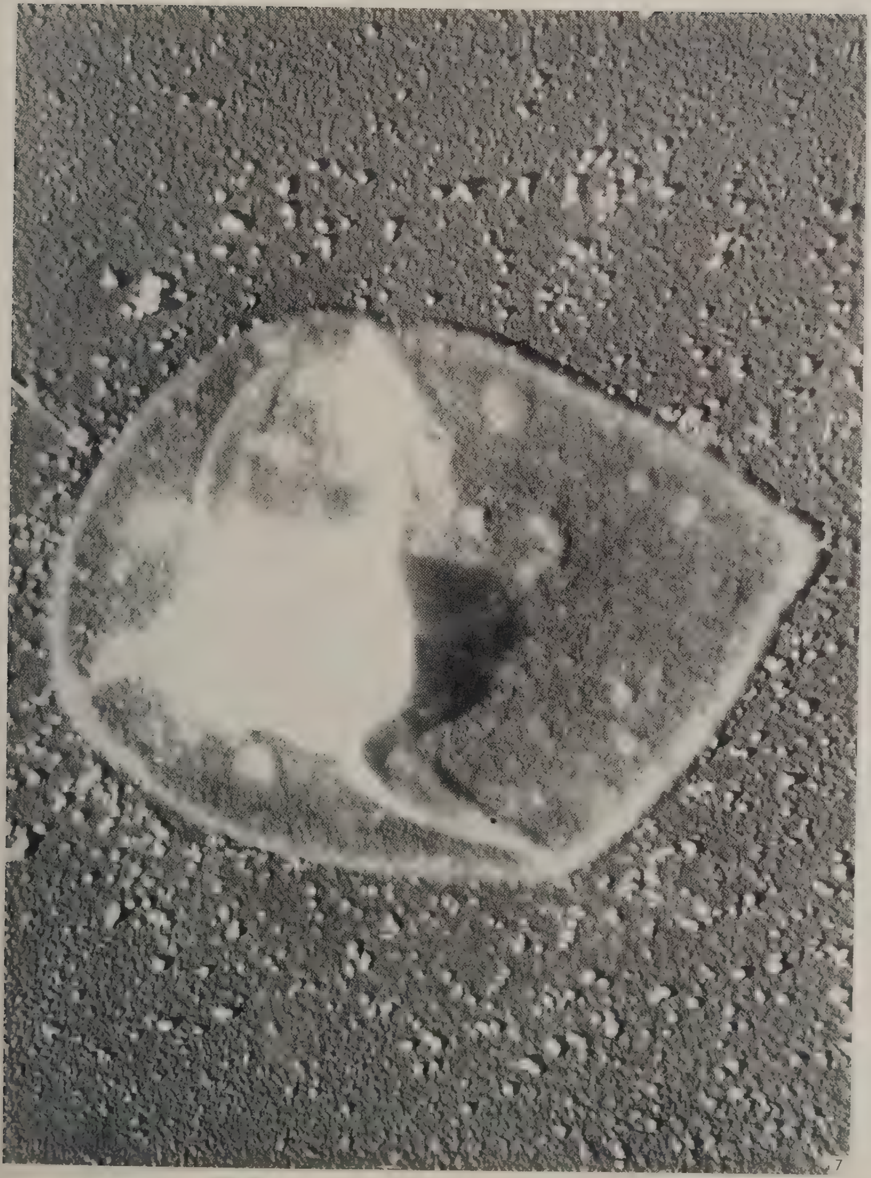
Little or no capsular material remained when *C. neoformans* was adsorbed from liquid culture medium onto the specimen membrane and then washed with water (Fig. 5). Similar results were obtained when the cells were washed in water by centrifugation prior to preparation of the specimen. The cells of *C. neoformans* shown in Fig. 5 had definite transparent cell walls similar to those found in water-washed cells of *H. capsulatum* (Fig. 2). Desiccation in high vacuum and exposure of directly adsorbed cells to electrons usually did not prevent dissolution of the capsule when washed with water. This is in contrast to the commonly held opinion that the electron beam fixes most biological material.

Shaking of liquid culture of *C. neoformans* with two volumes of ethyl-ether and subsequent washing of the cells with water by centrifugation (Fig. 6) resulted in partial removal of the capsular material and, as had also been observed with *H. capsulatum*, in apparent loss of protoplasm. The ability of the electron microscope to detect very thin capsules was demonstrated by magnification of two of the cells of Fig. 6 (Figs. 7 and 8). When capsular material was absent, the surface of the cell wall and specimen membrane consisted of distinct fine granules (Fig. 7). These granular structures became obscured when covered with a fine layer of capsular residues (Fig. 8).

DISCUSSION

The yeast phase of *H. capsulatum* grown in liquid medium under the conditions specified does not possess a capsule. With the aid of the electron microscope, Kligman and Baldrige [2] also failed to demonstrate a capsule of *H. capsulatum* on cells obtained from infected mice or grown on blood agar. However, their method of specimen preparation might not demonstrate a capsule even if such a structure were present. Comparative studies of encapsulated cells of *C. neoformans* showed that the procedure used by these authors of suspending unfixed cells in water would remove capsular material.

Fig. 7. *Cryptococcus neoformans*. Enlargement of lower left cell of Fig. 6. Magnification: 48,000 ×.



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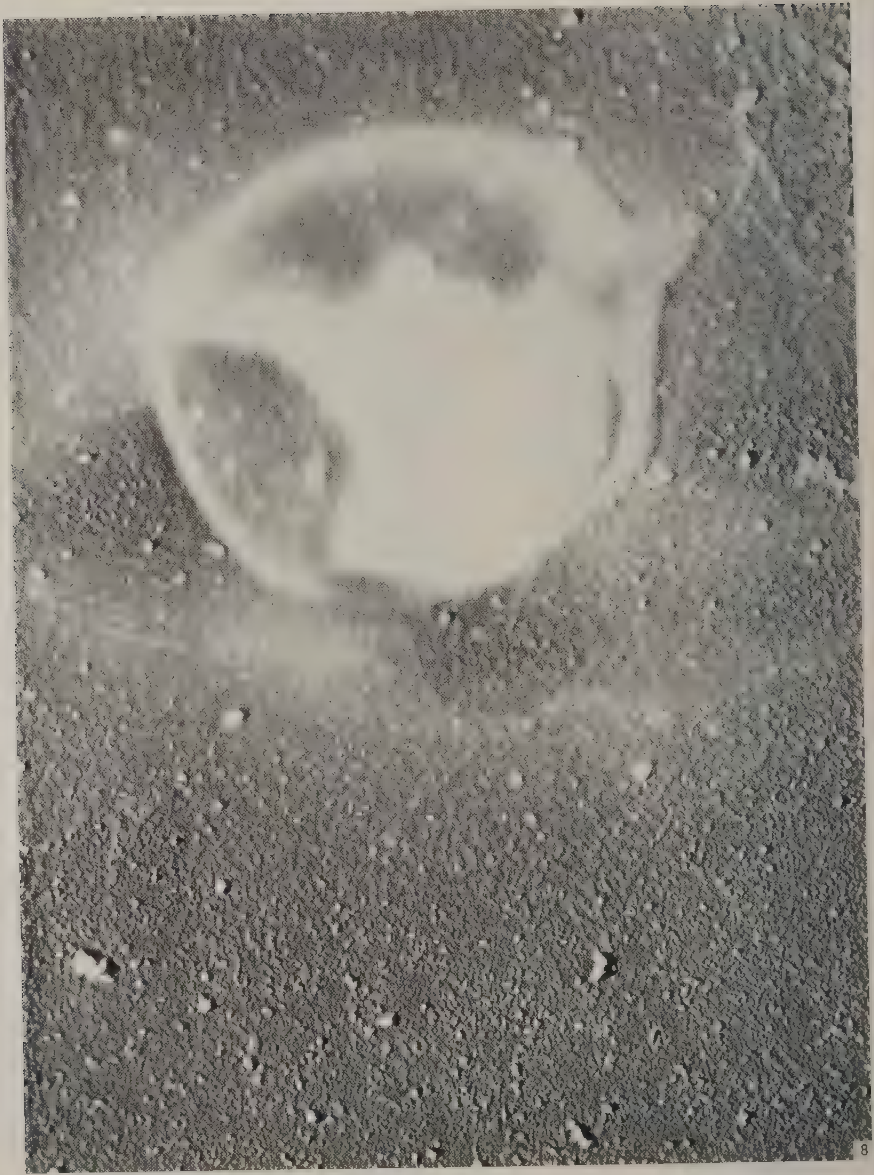
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The method of specimen preparation used here included the adsorption of cells from the original culture medium onto the specimen membrane, fixation in osmic acid vapor and removal of medium residues with water (Fig. 3). With this technique, the only uncontrolled factor that could cause artefacts was the desiccation of the cells upon the specimen membrane. This conclusion is based on observations of cells of the yeast phase adsorbed from the liquid culture medium without fixation or purification (Fig. 1). The micrographs of cells prepared by direct adsorption (Figs. 1 and 3) also revealed that transparent cell-wall zones of *H. capsulatum* and *C. neoformans* were not present. Therefore, the cell-wall zones usually seen in preparations of *H. capsulatum* and *C. neoformans* occurred because of preparation procedures involving treatment with water. The osmotic pressure of the environment changed during such treatment and a swelling of the cell wall occurred. The typical cell-wall borders were then formed upon the specimen membrane when the water-swollen cells were desiccated.

From the electron micrographs of *C. neoformans* (Figs. 4 and 6), it must be concluded that in liquid culture the cell body was surrounded by a capsule which was a nonintegral part of the cell and which existed as a cloud of enveloping molecules, the concentration of which decreased as the distance from the cell increased. Such a structure, therefore, observed after desiccation under the electron microscope, would not reveal a definite margin.

Micrographs of *C. neoformans* with most of the capsular material removed (Fig. 8) demonstrated the efficiency of the electron-microscopic method for the detection of very thin capsular layers. This efficiency was particularly noticeable when special attention was given to the method of shadow casting. Thus, the use of a metal alloy of high scattering power resulted in a film, the structure of which did not exceed the resolving power of the electron microscope or obscure the finest details of the preparation. Even the thinnest amorphous layers of the capsular material were detected when they obscured the small granules of the supporting film.

With the enhancement of the contrast of the minute details of the preparation surface by means of a structureless film of metals of high atomic number, it is possible to identify amorphous material of an even lower electron scattering power than the capsular substance of *C. neoformans*. This identification can be made without increasing the thickness and density of the capsule by means of homologous serum or binding with metallic salts [3, 4, 5].

Fig. 8. *Cryptococcus neoformans*. Enlargement of lower right cell of Fig. 6. Magnification: 48.000 ×.

SUMMARY

Comparative studies with the electron microscope were made of the yeast-like cells of *Histoplasma capsulatum* and *Cryptococcus neoformans*, grown in liquid medium. A capsule or slime layer could not be demonstrated on cells of *H. capsulatum*. *C. neoformans*, which had a distinct capsule, was used to indicate the sensitivity of the method for detection of extremely thin capsular layers. The importance of the technique used in the preparation of specimens for study of capsular substances was demonstrated.

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STUDIES OF THE METABOLISM OF B-VITAMINS AND RELATED COMPOUNDS IN EMBRYONIC SEA URCHIN DEVELOPMENT

I. PANTOTHENIC ACID AND LACTOBACILLUS BULGARICUS FACTOR

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From quantitative microbiological determinations of the changes in amino acid fractions, Kavanau [13, 14] has recently obtained information bearing upon the embryonic protein metabolism of the sea urchin *Paracentrotus lividus.* Periods of synthesis and degradation of proteins to and from free amino acids and peptides have been detected. In their most intense phases these processes appear to be more or less sharply set off from one another so that the changes in the amino acid fractions are of a complementary wave-like character. These changes are believed to be caused by the mobilization of amino acids and peptides from the yolk-protein reserves and their utilization in the synthesis of the proteins of the developing embryo.

Since several B-vitamin type factors were found to be present in microbially available form in the embryos of the same species [3, 4], the integration of the vitamin and amino acid studies seemed desirable. For microbiological studies of many of the B-vitamins special extraction procedures are required to liberate bound activity or avoid destruction of labile activity. Thus, in an exhaustive study of microbially detectible B-vitamin activity in the embryonic sea urchin it would be necessary to use several vitamin extraction procedures. The extraction techniques used by Kavanau were developed primarily for studies of amino acids. Nevertheless, because of the convenient opportunity offered for determining the developmental changes in B-vitamin activity and correlating these changes with those of the amino acids, a preliminary program has been initiated using the same extracts.

The results of these studies reveal that remarkable correlations exist between the various vitamin activities of these extracts and the changes in the amino acids. A comprehensive interpretation of these correlations will have to wait upon more detailed studies. However, it seems clear from our preliminary findings that the participation of vitamins in embryonic metabolism is generally reflected by quantitative changes in their stable or extractable forms. These changes appear to reflect changes in the extent to which vitamins or their coenzymes participate in morphogenetic reactions. The study of these changes provides a new and unique sort of approach to the problem of vitamin function and to the elucidation of the mechanisms of cellular control over metabolic processes.

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The pantothenate group of factors is widely distributed in living organisms and has become a focal point of interest because of the discovery that pantothenic acid is a constituent of coenzyme-A [9, 19].[1] Coenzyme-A (CoA) has been regarded as the sole metabolically active form of pantothenate. This is partly because no other biochemical function than vitamin and growth factor activity can be attributed to pantothenic acid (PA) or Lactobacillus Bulgaricus Factor (LBF), and partly because most or all of the pantothenate present in tissues was found to be firmly bound in a complex which was later identified as CoA [27] (CoA is unable to replace pantothenate for most microorganisms). Autolysis or clarase and papain treatment only partly release pantothenate from CoA, whereas combined splitting with alkaline phosphatase and avian liver enzyme results in complete release [21, 25].

The finding that aqueous extracts of steamed sea urchin egg homogenates had pantothenate activity for several lactic acid bacteria [3] led to tests for free PA and LBF activity in the quantitative acetone–HCl extracts of the same material. In this paper we present the results of assays for the free pantothenate fraction.

MATERIAL AND METHODS

Test material.—Aliquots of the same extracts of *P. lividus* eggs and embryos prepared by Kavanau [14] for his amino acid determinations were employed for these studies. The extracts were obtained from 22 developmental stages (unfertilized egg to 50-hour pluteus) and 3 "stages" of aged unfertilized eggs. The material was collected on an "equal number of embryos" basis to avoid errors inherent in other methods of baseline determination [12, 14].

Extracts.—The operations used in collecting the material and preparing the extracts are given in detail in previous papers [12, 14]. The extraction techniques were originally developed for studies of amino acid fractions. Although we do not possess data concerning quantitative aspects of this extraction of pantothenate from the sea urchin material, we believe that a rather good recovery of the free pantothenates (which are actually relatively small heteromeric peptides) was achieved.

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[1] The following conventional abbreviations for pantothenate compounds are used in this paper: pantothenic acid = PA; Lactobacillus Bulgaricus Factor of Snell = LBF [34] (pantethine = PSSP, pantetheine = PSH [5]); Pantothenic Acid Conjugate of Cheldelin = PAC [15]; coenzyme A = CoA.

One possible limitation of the extraction procedure might be the low pH of the acetone–HCl extracts during certain phases of the operation. This could conceivably be responsible for loss of pantothenate, often reported to be sensitive to acid or alkali. However, very little of a detailed nature is known about the acid sensitivity of PA [28] or LBF. According to Lepkovsky [17] PA "is quite stable in neutral solution, but its activity is destroyed by hot acidic or alkaline solutions" because of the splitting of the peptide bond. However, different forms of PA and LBF probably behave rather unconventionally and are protected in chemically complex surroundings. It was found, e.g., that naturally occurring PA is partly protected, at least against alkali, owing to the presence of the phosphorylated forms in tissues and fresh material [11, 16, 2]. In the case of the sea urchin material, the compounds in question may have been protected by maintaining the extracts at 0° C or in the frozen state during processing.

Microbially active pantothenate might conceivably be liberated enzymatically during the course of extraction [cf. 18, 32, 21]. However, it should be kept in mind that the present extraction conditions were unfavorable for autolysis and enzymatic release of PA from CoA.

Test organisms.—*Lactobacillus casei* (ATCC 7469), *Lactobacillus delbrückii* 730 (ATCC 9649) and *Lactobacillus acidophilus delbrückii* (ATCC 4796) were selected on the basis of the preliminary cup plate work [3].

L. arabinosus was judged unsuitable for the present work because it gives hardly any response with pantothenate in the cup plate test, in spite of good growth in liquid medium. Moreover, *L. arabinosus* was reported to utilize LBF to a much lesser extent than PA [8, 20] and, in fact, very little pantothenate activity was found in the aqueous embryonic extracts with *L. arabinosus* [3].

Media.—The uncompleted medium of Skeggs and Wright [31] served for *L. casei* (Bacto Pantothenate Assay Medium). This medium was completed for *L. delbrückii* and *L. acidophilus* according to Bánhidi [3] as noted.[1]

Assay.—The semi-micro assay technique of Kavanau [12, 14] was adapted for the pantothenate test. Inocula were prepared by washing a 24-hour Bacto Micro Inoculum Broth culture twice in saline and suspending at a dilution corresponding to about 1 to 200 of the original growth (standardized turbidimetrically). The *L. casei* inoculum was diluted only 1 to 40. One volume of these suspensions was dispersed in 30 volumes of sterile (autoclaved for 10 minutes at 10 pounds pressure) double-strength medium. The samples and standards were sterilized separately by repeated freezing and thawing (–15 to +10° C) instead of the conventional autoclaving generally accepted for routine assays. This precaution was taken to obviate the destruction of pantothenate (which is especially labile in the standards because of the lack of the protecting effect of natural extracts), or to prevent the liberation of pantothenate from bound forms. After incubating for 45 to 51 hours at 35°C the growth was halted by steaming for 15 minutes and titrated as previously described [12].

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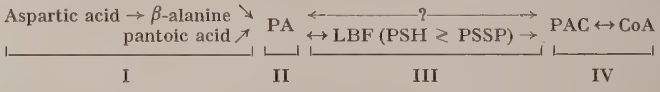
[1] Alkali-treated peptone, 20 ml; Norite-treated Bacto Yeast Extract, 2 ml; Tween 80, 0.1 g; DL-alanine, L-asparagine and L-cysteine, 10 mg each; xanthine, 1 mg; thymidine, 0.1 mg; ascorbic acid, 0.1 g; pyridoxal and pyridoxamine, 10 μg each; folic acid, 2 μg; vit. B_{12}, 0.5 μg; and ammonium sulphate 30 mg, per 100 ml of double-strength basal medium.

Standard solutions of PSSP[1] (generally compared with Ca–D-pantothenate) were used in triplicate in each assay series at 8 different concentration levels, ranging from 1 to 25 mμg per ml final volume. Samples of egg extracts were determined in duplicate or triplicate as material permitted.

The nature of the compounds assayed.—In contrast to many yeasts, which utilize factors of groups I and II, the majority of the Lactobacilli grow only in the presence of factors II or III. *Acetobacter suboxydans* prefers the –SH forms of groups III and IV. However, factors IV seem to be unable to enter cells of the Lactobacilli used in these studies and therefore do not interfere with the assays [8, 32, 6]. According to Craig and Snell [8] the PA activity coefficients for both synthetic PSSP and natural LBF were approximately 1, 130, and 430 for *L. casei*, *L. delbrückii* and *L. acidophilus*, respectively, if measured turbidimetrically after 18 hours of incubation. McRorie and Williams [20] found the ratios of free pantothenate activity to that of an LBF concentrate to be 1 and 167 for *L. casei* and *L. acidophilus* (4796), respectively, using essentially the same procedure.



In our work, the final acid production was used because of limitations imposed by the minute amounts of extracts available. Under these circumstances the early growth stimulation with LBF is not recorded. PA was found to be only 42 per cent as active at a concentration corresponding to 10 m μg PSSP per ml, while 70 per cent activity was found at the 4 mμg per ml level, if values are calculated in mole equivalents of PSH. This is because the slopes of the response curves for the two factors are different, as was indicated by the cup plate tests [3].

Thus, the compounds tested by us and expressed in pantetheine (PSH) units should be free forms of PA and/or LBF, both of which are known to occur in several combinations (possibly mixed disulfides) in natural materials [7, 6]. Neither pantoic acid nor the pyrophosphate of LBF (PAC) nor CoA should interfere with our test.

RESULTS

The curves obtained with all three organisms are essentially identical in their appearance, although the absolute values of the activity calculated relative to PSH vary somewhat with the organism used. This difference probably results because of the different sensitivities of the assay organisms to the active compound(s) present in the extracts, although the higher values obtained with *L. casei* may be due to the fact that the assay medium used is less complete for this organism as regards stimulatory factors, e.g.

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[1] We are indebted to Dr. H. M. Crooks, Jr., of Parke Davis and Co., Detroit, Mich., for the sample of pantethine, Lot No. 1918 × 66.

strepogenins. The curve obtained by averaging the results for all three organisms is given in Fig. 1. The curves for the protein and non-protein amino acid fractions are included for comparison. The absolute values obtained with each organism (average of two closely checking assays) are tabulated in Table I.

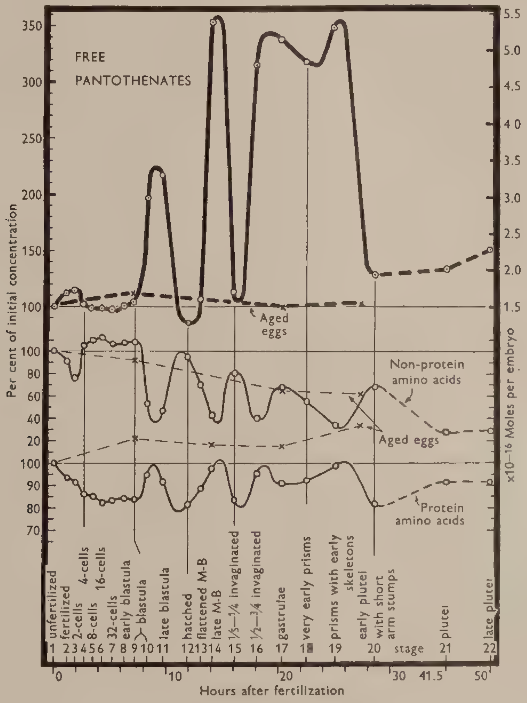

Fig. 1. Developmental changes in free pantothenate activity. The curve represents the average of assays with *L. casei*, *L. delbrückii* and *L. acidophilus*. The left ordinate scale gives the percentage of the activity or concentration in the unfertilized egg while the right ordinate scale gives the mole-equivalents of pantetheine.

From comparison of the curves in Fig. 1 it is evident that the marked increases in pantothenate activity occur roughly at periods when protein synthesis and depletion of the non-protein amino acids take place.

The first appreciable increase in pantothenate activity to stage 10 occurs through a period when protein synthesis takes place. However, the activity is even greater at stage 11 when protein synthesis appears to have ceased and yolk-protein degradation appears to be in progress. This would suggest that the free pantothenates are either involved in ancillary reactions concomitant with both protein synthesis and degradation, or that the reactions in which they are involved merely lag behind those directly concerned with

TABLE I

Free pantothenate content of *P. lividus* embryos and eggs (10^{-16} moles/embryo equivalent to pantetheine).

Stage*	Assay organism		
	L. casei	*L. delbrückii*	*L. acid. delbrückii*
1	1.71	1.61	1.25
2	1.95	1.78	1.36
3	1.91	1.82	1.46
4	1.75	1.50	1.33
5	1.79	1.49	1.23
6	2.06	1.18	1.23
7	1.85	1.34	1.24
8	2.06	1.26	1.29
9	2.10	1.24	1.42
10	3.10	2.27	2.58
11	3.74	2.98	3.18
12	1.55	1.10	1.23
13	1.97	1.50	1.38
14	5.26	4.86	5.98
15	2.06	1.66	1.37
16	5.04	4.39	4.93
17	5.53	4.45	5.44
18	5.02	4.37	5.11
19	5.57	4.83	5.46
20	2.76	1.27	1.78
21	2.80	1.65	1.62
22	3.14	1.95	1.74
Aged 7 hrs.	1.98	1.68	1.41
,, 20 hrs.	1.67	1.60	1.25
,, 27 hrs.	1.78	1.62	1.28

* For explanation see Fig. 1.

protein synthesis; beginning later and ending later, with any overlap with yolk-protein degradation being coincidental. The latter alternative is apparently supported by the fact that the activity is low at stage 13 but very high at stage 14. A wave of protein synthesis begins at about stage 12, continues intensely through stage 13 and terminates shortly before or after stage 14. Apparently the reaction(s) in which the free pantothenates participate has barely begun by stage 13 but has reached full intensity at stage 14. The activity is also intense at stages 16, 17, 18 and 19 but the amino acid metabolism through this period is not definitely established, it being only possible to state that protein synthesis occurs between stages 15 and 16, and stages 18 and 19. The activity is low but shows an increasing trend from stages 20 to 22. Although protein synthesis occurs at some time between stages 20 and 21, the determinations are at relatively great intervals so that correlation between the two curves is uncertain.

There appears to be a slight increase in pantothenate activity at stages 2 and 3. If this increase is real it might also be associated with protein synthesis, for an increase in soluble protein occurs in the interval between stages 1 and 5 (see [14] or Paper II of this series). There are only slight increases in pantothenate activity in the aged eggs in which yolk-protein synthesis is believed to occur [14] but these changes also correlate with those of the amino acids of the protein fraction. The low magnitude of these changes may reflect a lesser participation of pantothenates in reactions concomitant with yolk-protein synthesis, or they might be explained by the fact that the determinations are too widely spaced, so that periods of high pantothenate activity have escaped detection.

It is clear that much more closely spaced determinations would be required in order to make possible a detailed analysis of the relationship between the changes in amino acids and pantothenate activity. It is not possible to decide upon the basis of these data whether the free pantothenates of the sea urchin embryo are independent of CoA metabolism or are, in accord with general belief, involved only in CoA metabolism. According to Lipmann, "it appears unlikely that there is another pantothenic-acid containing coenzyme in animal tissues" [18]. After the latter interpretation, the pantothenates of the sea urchin embryo should function in acetylation reactions, involved primarily in carbohydrate oxidation. The involvement of these factors in protein metabolism is also possible if CoA is concerned in the synthesis of some of the amino acids by way of its participation in ketoglutarate and citrate metabolism. An involvement of PA in protein metabolism was suggested several years ago by the finding of severe impairment of antibody response in PA-deficient rats [1].

DISCUSSION

A change of assayable free pantothenates might be due to their synthesis from or breakdown into precursors [30, 29, 33]. Alternatively this might be caused by the binding and release of pantothenates from states in which they are not microbially active in our assays.

It is not likely that the first consideration obtains. According to general experience the animal body is dependent upon an exogenous supply of pantothenic acid (PA) [22, 30]. The vitamin character of PA has not yet been demonstrated for the sea urchin. However, it is not very likely that the sea urchin would present a special case insofar as the vitamin character of PA is concerned. In fact, the synthesis of PA is known to occur only in plants and microorganisms [29, 33, 22]. Its enzymatic breakdown has not been extensively studied in plants or in animals. Thus, the first possibility of PA synthesis by the sea urchin embryo can be dismissed.

In animals PA has been found to occur almost exclusively in the bound form as CoA. The only exception seems to be the blood of mammals where no CoA has been found, although 30 to 50 μg per cent PA is present [cf. 23, 22, 35, 26]. The synthesis of CoA from PA in the blood is believed to be brought about by the different organs according to their needs. The steps in the synthesis of CoA are known from the work of Novelli *et al.* [24, 25, 22, 23, 10].

With regard to present knowledge, reversible reactions which result in synthesis or breakdown of CoA might be the cause of the changes which occur in the free pantothenate content of the sea urchin embryo. The content of CoA might, in fact, be regulated by such a process. In certain phases of development a greater amount of CoA might be needed, whereas in other phases a lesser amount might correspond to the requirements. Accordingly the amount of free pantothenates in the embryos would be expected to undergo changes. In this connection the unfertilized egg acts as a sort of physiological control which aids in the interpretation of these changes. The egg is in a more or less resting condition before fertilization and through the period of aging when its free pantothenate content is very low. At these times its metabolism is primarily that of maintenance, and a cross-section of the concentrations of most metabolites would probably reflect a condition of minimum metabolic participation. Regarded from this point of view the indication is that when the free pantothenate content of the embryo is low the extent of participation of pantothenates in morphogenesis is also low, while an increased free pantothenate content corresponds to an increased participation. This condition would result, for example, if CoA were in equilibrium with the free pantothenates. This relationship is also suggested by the fact that, whereas great increases in free pantothenates occur in development, their content scarcely ever falls below the value in the unfertilized egg but rather shows an increasing trend with development.

Finally, we cannot disregard the following possibility. Pantothenates might be bound in the sea urchin embryo in such a way that they withstand extraction by the method adopted in the present work. The changes in free pantothenate content might then be caused by transitions from soluble pantothenates to insoluble ones and vice versa. The soluble pantothenates might serve as precursors for CoA. The data available at present do not permit deciding between these alternative interpretations. The continuation of this series of investigations will probably cast further light on this problem.

SUMMARY

Changes in free pantothenates were studied in acetone–HCl extracts of 22 stages of embryonic development and 3 "stages" of aged unfertilized eggs of the sea urchin *Paracentrotus lividus*.

Similar results were obtained with all three bacteria used for test assays (*L. casei*, *L. delbrückii* and *L. acidophilus*). A marked increase in the free pantothenates occurs at certain times in development (up to 3.6 times the original amount). The increases occur roughly during periods when new embryonic protein synthesis takes place. Pantothenates (probably bound as CoA) may participate in reactions synchronized with but lagging behind those directly involved in protein synthesis.

The results suggest that in the sea urchin embryo the supply and withdrawal of free pantothenates may be a regulatory mechanism for reactions in which they or CoA participate. The implication is that the rate of some CoA dependent enzymatic processes might be controlled by the concentration of this coenzyme, which may itself be in equilibrium with the free pantothenates.

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STUDIES OF THE METABOLISM OF B-VITAMINS AND RELATED COMPOUNDS IN EMBRYONIC SEA URCHIN DEVELOPMENT

II. THE NIACIN GROUP OF FACTORS

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In the first paper of this series [3] the quantitative developmental changes of free pantothenates in extracts of *Paracentrotus lividus* eggs and embryos have been reported. Analysis of these changes and their correlation with the amino acid metabolism [18] suggests that free pantothenates (and/or coenzyme–A) participate in reactions synchronized with but lagging behind those directly involved in new embryonic protein synthesis.

In this paper we report the findings for the niacin (nicotinic acid) group of factors. Almost all the activity of this vitamin group in animal tissues is claimed to be due to nicotinamide (nicotinic acid amide) bound mainly in pyridine nucleotide coenzymes [11, 27, 1, 15]. The only metabolic role of nicotinamide hitherto established is its function at the cellular level as a moiety of the coenzymes: codehydrogenase I (DPN = diphosphopyridine nucleotide = Co I = cozymase), codehydrogenase II (TPN = triphosphopyridine nucleotide = Co II) and cysteine sulfinate dehydrogenase [31], also called Co III (nicotinamide-ribosepyrophosphate) [11, 38, 15, 32, 34]. The free nicotinamide of body fluids or parenterally administered niacin is generally regarded as serving solely as a precursor for coenzyme synthesis in the tissues.

Niacin, nicotinamide, DPN, TPN and some other derivatives are known to be equally active (on a molar basis) in the nutrition of *Lactobacillus arabinosus* 17-5, the assay organism used for the present studies. Of these active factors, the free niacin compounds were analysed by excluding the intact coenzymes from the extracts of the sea urchin material.

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EXPERIMENTAL

Material and extraction methods.—Aliquots of the same acetone–HCl extracts of *P. lividus* eggs and embryos prepared by Kavanau for his amino acid studies [18] and used by Bánhidi and Kavanau [3] for determinations of the free pantothenates were employed.

Microbiological assays.—The semi-micro assay technique already described [17, 18, 3] was adapted for the assay of the niacin group. Assays were carried out with *L. arabinosus* 17-5 (ATCC 8014), the organism widely used for the determination of niacin [33] and chosen for the present work on the basis of preliminary cup-plate tests on aqueous extracts [2]. The basal medium of Barton-Wright [5] (Bacto Niacin Assay Medium) was completed with preparations used by Sarett *et al.* [28] in the same way as for the cup-plate tests[1] [2]. A standard curve was obtained using serial dilutions of nicotinamide in the range 0 to 200 mμg per ml final volume. The inoculum was prepared as before [3] and added at about a 1 to 250 dilution (one volume of this dilution to 30 volumes of double-strength medium). The samples, standards and medium were sterilized by autoclaving at 10 pounds pressure for 10 minutes. After incubating for 40 to 48 hours at 35° C the growth was halted by steaming for 15 minutes and the cultures titrated as previously described [17].

Evaporation during incubation is greatly reduced by covering the tubes with a double layer of aluminum foil held firmly in place by the flexible cotton-in-gauze lining of the rack-lid. After sterilization, one layer of foil is temporarily removed and the medium and inoculum are added (with a syringe-pipette) by piercing the remaining layer of foil.

The scope of the assay.—A large number of specific dehydrogenases exist. These consist of pyridine nucleotide coenzymes combined with different substrate-specific apoenzymes [34, 29, 32]. The degree of association of the coenzyme with the apoenzyme is, in fact, less than for any other B-vitamin-containing coenzyme. The "mobility" is greatest in the reduced form [36].

Because of this mobility of the coenzymes, the extraction of the niacin group of factors from natural materials can be carried out in a relatively simple way, if one wishes to determine only the total group activity. A large part of the activity can be extracted by simply autoclaving the material with water [35]. In some cases where this does not effect quantitative removal, the material is more commonly autoclaved in 1*N* acid [27, 33]. More drastic alkali liberation of nicotinamide from its alkyl esters is required only in the case of some cereal products [20].

The procedures used in the present studies were developed primarily for the isolation of amino acid fractions [18]. The effect of the extraction technique on the release and extraction of the niacin factors was not studied in detail. However, on the basis of the available chemical data the following account can be given of the behavior of the niacin factors found in the extracts of sea urchin eggs and embryos.

The first treatment of the lyophilized material with 1.1M KCl – 0.011M HAc (thrice for 24-hour periods in the ice-cold solvent) should extract the dissociated pyridine nucleotide coenzymes and freely occurring members of the niacin group.

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[1] *Special supplement*: 20 ml "Lloyd's Reagent-treated peptone, liver and yeast mixture" and 8 ml "Norite-treated yeast extract" per 100 ml double-strength medium [28, 5].

In disrupted cells the nicotinamide coenzymes are relatively unstable because of enzymatic cleavage [cf. 10, 37, 29, 15]. Enzymatic "inactivation" of DPN and TPN can take place to some extent during both yeast and muscle "brei" extraction, if conditions are favorable. Deamination of the intact coenzyme or splitting of the phosphate bridge are the main processes involved in this "inactivation" [cf. 24, 15]. However, according to the studies of Euler *et al.* [10] on the enzymatic splitting of cozymase (no cleavage at or below pH 5 when incubated for 18 hours at 30° C), the enzymatic liberation of nicotinamide from its coenzymes in the sea urchin extracts can be excluded. Both the acidity (pH 4.5) and the low temperature of extraction were unfavorable for such enzyme action.

The extracts described above were lyophilized and extracted several times with 5 per cent $0.5N$ HCl in acetone by first suspending in one volume of ice-cold $0.5N$ HCl and subsequently adding 19 volumes of acetone. Acetone is one of the best possible solvents for the extraction of nicotinamide and free forms of related compounds. Acetone also blocks the activity of cozymase splitting enzymes [12]. The pyridine nucleotide coenzymes are soluble in 50 per cent acetone [26, 37] but precipitate when the concentration of acetone is increased to 80 per cent or above [26]. This makes it possible to completely separate the coenzymes from the smaller molecules of the niacin group [12]. Thus, the acetone-HCl extracts should contain practically all the free niacin compounds and little if any nicotinamide coenzymes.

The oxidized forms of the pyridine nucleotides are relatively stable to chemical and physical treatments [24]. However, their reduced forms are "rapidly inactivated" under acidic conditions [37, 11]. These reduced forms are known to be present in animal tissues in a fairly constant percentage (35 to 40 per cent of the total) [11, 25]. They are, however, rapidly oxidized by contact with atmospheric oxygen during extraction, except under alkaline conditions [11]. The acidic "inactivation" of the reduced forms was interpreted by Karrer and co-workers [16] as an irreversible addition of mineral acids to the double bonds of the pyridine ring of the cozymase molecule, and the resulting inactive coenzyme was found by Euler *et al.* to show much less nicotinamide activity when hydrolyzed, extracted and assayed colorimetrically [11]. In the light of these findings, the acidic "inactivation" of the reduced coenzymes, which probably occurs during both extractions of the sea urchin material, would not result in actual liberation of nicotinamide or related forms from the coenzymes. Thus, the overall picture for the free niacin group should not be affected.

In view of the above considerations we are probably justified in concluding that the pyridine nucleotide coenzymes are eliminated from the final extracts. The activity represented by the niacin assays of these extracts should be due primarily to unaltered free factors of the niacin group. Only a negligible fraction of the activity is probably due to the coenzymes or to nicotinamide having its origin in their breakdown.

RESULTS

The curve showing developmental changes in the total activity of the free members of the niacin group is plotted in Fig. 1 (per cent of the value in the unfertilized egg). The curves for non-protein amino acids and the amino acids of the soluble (KCl–HAc) and insoluble proteins are given for comparison. The qualitative pattern of changes for the activity of this vitamin group has been confirmed repeatedly and the curve represents the average of two assays in the best range of the standard curve. The ordinate on the right expresses the activity in moles per embryo relative to nicotinamide standards.

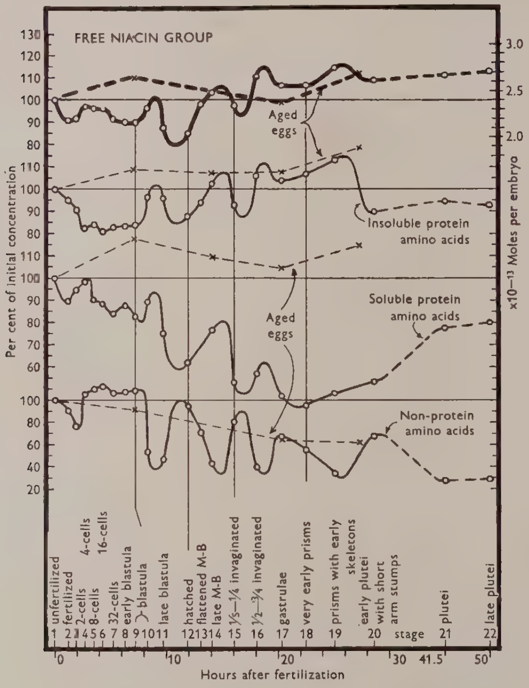

Fig. 1. Developmental changes in the free niacin factors. The left ordinate gives the percentage of the amount in the unfertilized egg, the right ordinate gives the activity in mole-equivalents of nicotinamide. The amino acid curves are given for comparison.

Offhand, one would not have expected the total activity of the free members of the niacin group to show a close correlation with the changes in any other single group of metabolites. By reference to the curves of Fig. 1, however, it can be seen that the changes in the free vitamin activity are strikingly similar to those of the amino acids of the insoluble-protein fraction. Not only is the same general pattern of cyclic changes found, but the magnitude of the changes is about the same and the same trend of increase in concentration from stage 9 to stage 19 occurs. This contrasts with the opposite trend in the amino acids of the soluble-protein fraction (although the general pattern of cyclic changes is the same) and with both the opposite trend and the complementary

TABLE I

Concentrations of B-vitamins (mole-equivalents of the corresponding standards) compared with valine.

Substance (and standard)	Concentration (10^{-16} moles/embryo) and per cent of the value in the unfertilized egg							
	Unfertilized egg		Minimum value	Per cent	Stage	Maximum value	Per cent	Stage
	Value	Per cent						
Free pantothenates (pantetheine)	1.52	100	1.29	85	12	5.37	353	14
Free niacin factors (nicotinamide)	2,400	100	2,040	85	12	2,720	113	22
Non-protein valine (valine)	78,000	100	12,500	16	20	94,000	115	6
Protein valine (valine)	439,000	100	331,000	75	21	445,000	101	14

cycles of the non-protein amino acids. On the other hand, certain changes in the activity of the free niacin factors resemble changes in the soluble proteins more than they do those in the insoluble ones (see stages 2 to 4, 10 to 11, and the changes from 7 to 20 hours of aging unfertilized eggs).

The curve for free niacin factors possesses an early cycle between stages 2 and 8 which is not found in the curve for the insoluble-protein amino acids. Because of the otherwise close similarity between these curves, this could be regarded as an indication that an undetected cycle occurs in the insoluble proteins at that time. This was suspected on other grounds [18]. If such a cycle occurs it may not be revealed by the amino acid determinations because it is eclipsed by the great wave of yolk-protein degradation occurring at the same time.

When unfertilized eggs are allowed to age, protein synthesis occurs and the non-protein amino acids are depleted (see Fig. 1 and ref. [18]). The changes in the activity of free niacin factors through this period also parallel those of the insoluble proteins. Thus, it is unlikely that the relatively sharp increase in the insoluble proteins between 20 and 27 hours of aging (and also found in the niacin activity) is an artefact caused by precipitation and consequent collection of the egg jelly as was suggested [18]. The results obtained with other vitamin groups [cf. 3, 4] support this interpretation.

Calculated as nicotinamide, the molar concentration of the niacin-group activity is 300 to 1500 times as great as that of the other vitamin groups we are investigating [cf. 3, 4]. This activity corresponds to about one molecule of nicotinamide to 4 to 8 molecules of protein in the sea urchin embryo (approximately one molecule of nicotinamide to 150 molecules of each of the amino acids in the protein fraction).

In Table I we have tabulated the concentrations of the vitamins (activity in mole-equivalents of the standards) thus far reported (see Paper I of this series) and their maximum fluctuations from the values in the unfertilized egg. The concentrations of free and protein valine [18], a typically behaving amino acid, are given for comparison.

INTERPRETATIONS AND DISCUSSION

It is well known that the amount of nicotinamide present in tissues greatly exceeds that of the other B-vitamins. Thus, Williams *et al.* determined the total vitamin content per tissue and found that on a weight basis the B-vitamins were present in the following order of abundance: niacin >pantothenate >riboflavin >thiamine >biotin (cf. [38], pp. 23–29). It has also been recognized that on an equivalent basis the pyridine nucleotide coenzymes occur in quantities which are considerably in excess of the amounts of their apoenzymes. This situation is believed to be partly accounted for by the fact that the coenzymes are mobile and must alternate between two separate apoenzymes (cf. [38], pp. 137–138) and partly by the fact that in "reduced enzymes" 3 moles of DPN are bound to each mole of enzyme [22]. However, our determinations in the closed developing system represented by the sea urchin embryo show that the disparity between the concentration of the free niacin-group factors and those of other vitamins is far too great to be explained in this way. In the case of the sea urchin, at least, the major portion of the vitamin activity we have determined is probably to be attributed to metabolites having some other physiological role than serving as precursors for pyridine nucleotide coenzymes. In fact, bioautographic studies we are now carrying out [4] have disclosed that the vitamin activity assayed by *L. arabinosus* is due to from 5 to 7 or more different compounds (at most one of which could be DPN or TPN), suggesting a great complexity in the metabolism of this vitamin group. In this connection, the variations in the free niacin-group activity are relatively small compared to those of most other vitamins we are studying (cf. Fig. 1 and refs. [3, 4]). On the other hand, large changes in the concentrations of microbially active metabolites of this group could occur without being detected by our assays for the total activity, for complementary changes might cancel one another.

The fact that the curve of free niacin-group activity bears a close similarity to the curve for the insoluble proteins appears to have no ready explanation upon the basis of the data at hand, especially as this curve represents the sum of the activities of a number of substances. However, there would seem to be some reason to suspect that the free niacin factors may be involved in some way in protein metabolism.

The curve for free niacin factors could include at most a very small component due to pyridine nucleotide coenzymes. However, since part of the activity represented by the curve may be due to metabolites serving as precursors for these coenzymes, a component of the changes might be caused by withdrawal and return of such precursors to the pool as the coenzymes were synthesized and degraded. In this connection it is known that the activity of the pyridine nucleotide coenzymes varies in the development of some embryos [23, 13]. However, the changes caused by this participation would probably be of relatively small magnitude.

On the other hand, the changes could reflect interconversions between microbially more and less potent derivatives in development. Thus, while niacin, nicotinamide, nicotinuric acid, DPN, TPN and split products of the coenzymes are equally active for *L. arabinosus* [cf. 36, 2], nipecotic acid possesses only slight activity and picolinic acid and trigonelline exert only minute activities in the cup-plate test [2]. Furthermore, homarine (picolinic acid methylbetaine) occurs in considerable amount in *P. lividus* embryos [14] and it is practically inactive for *L. arabinosus* [2].

The occurrence of nicotinamide synthesis from tryptophan in mammals and *Neurospora* is well known [21, 6, 7]. There is also *de novo* synthesis of both niacin and pyridine nucleotide coenzymes in the avian embryo [8, 13] where (under germ-free conditions) tryptophan seems to be the precursor [1, 30, 9]. It was not possible to correlate the changes in the free niacin factors of this series with those of free tryptophan. However, studies of the KCl-HAc extracts of another series [19] show that the changes in free tryptophan are the same as for the other free amino acids [18].

It will probably be possible to better assess the significance of the changes in the free niacin factors when the various compounds which are present in the sea urchin extracts are identified and their individual changes and relative activities determined. Further studies of this vitamin group are underway.

SUMMARY

Quantitative developmental changes in free niacin factors (largely excluding the pyridine nucleotide coenzymes) were determined by microbiological

assay (with *L. arabinosus*) of the same extracts of *Paracentrotus lividus* eggs and embryos used for the study of the free pantothenates (Paper I of this series).

The concentration of free niacin factors in the sea urchin is 300 to 1500 times greater than those of the other B-vitamins and far greater than is to be expected if its function is merely to contribute to the structure of the pyridine nucleotide coenzymes.

The curve for the developmental changes in the activity of this vitamin group is strikingly similar to that for the isoluble protein fraction, and the relative magnitude of these changes is small compared to those found for most other B-vitamins.

These findings suggest that the free niacin factors of the sea urchin embryo have some other physiological role besides serving as precursors for the pyridine nucleotide coenzymes.

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TRANS-FILTER INDUCTION OF TUBULES IN MOUSE METANEPHROGENIC MESENCHYME

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A NUMBER of hypothetical mechanisms to explain phenomena of embryonic induction have been proposed, but none has yet been sufficiently strongly supported experimentally to command general acceptance. It is generally assumed that inductive effects reflect some transmission between the two interacting tissues, but the intimacy of association usually involved has complicated the problem of identifying the effective agent. Several investigators have interposed barriers to direct tissue contact and have found inductive effects to be obliterated [4, 18, 20]. Others have reported weak [5] or positive [10, 23] results in widely different inductive systems. It seems clear that barriers to cellular penetration which permit inductive effects consistently to cross them would provide an "interzone" within which the actual effective agent might be identified in transit.

Materials previously tested for such barrier action have usually been low porosity membranes such as cellophane which might be expected to exclude high molecular weight compounds. On the other hand, highly porous filters of interlacing fibers would not be expected to prevent cellular penetration. The behavior of filters of intermediate porosity, so-called membrane or molecular filters[2] which exclude passage of bacteria but not of high molecular weight materials such as proteins, is the subject of this report.

The inductive system selected for study involves embryonic spinal cord and metanephrogenic mesenchyme of the mouse. Origin of the epithelial precursors of secretory tubules in nephrogenic mesenchyme has been extensively studied in amphibia [6] and the chick [16]and there is a strong consensus that it normally depends upon an inductive stimulus from the Wolffian duct or, in the case of the metanephros, from the ureteric bud which branches from the duct. It has been shown that embryonic spinal cord can also provide the inductive stimulus [16]. These general conclusions have recently been substantiated for the mouse in studies *in vitro* [12] and the additional finding has been made that tubule-inducing activity in the cord is highest in the dorsal half prior to the 13th day of gestation. The strong inductive activity of embryonic dorsal spinal cord when directly combined with metanephrogenic mesenchyme, together with the relative abundance of available spinal cord in comparison with ureteric bud, suggested dorsal cord as a favorable inductive source for trans-filter studies.

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[1] With the technical assistance of Gayle Bankard, Derrell Freese, George Parker and Edward J. Soban.

MATERIAL AND METHODS

Filters.—The general nature and properties of cellulose ester membrane filters, and their application to a number of microbiological problems, have been described [9]. The filters are commercially available in sheets of 150 micron thickness in two porosity grades, hydrosol (HA) and aerosol (AA). Data on the commercial filters, provided by the manufacturer[1], indicate that the substance consists of a "network of cross linked elements approaching molecular dimensions" and defining "uniform pores of a total volume equal to 80 to 85 per cent of the volume of the sheet". Performance data are said to suggest that the pore axes "may be considered for practical purposes as though perpendicular to the surface of the filter membrane". Calculated pore size is given as 0.45 micron for HA and 0.8 micron for AA filters. The manufacturer's data are supported by a recent study of pore-size distribution in standard HA-type filters using a mercury-intrusion method [19]. The data on two samples suggest a peak in pore size distribution at effective pore radii of 0.5 and 0.6 micron respectively, with some gradation below these values but a sharp cut-off above.

It is to be emphasized, however, that the material used in the experiments to be described was *not* the standard 150 micron thickness filter, but specially manufactured thinner sheets. These varied somewhat in thickness, measuring 20 ±4 microns for the HA-type, and 20 ±4 and 30 ±2 microns for the AA-type. For convenience, samples from these several sheets, despite thickness variation, will be referred to as 20H, 20A and 30A, respectively. The thinner sheets were chosen to bring the two tissues into closer association, and because their translucency when wet provided greater operative and observational convenience. Their use, however, is subject to the disadvantage that no physical data are available on their porosity, and there is reason to believe from the biological data that their porosity is not the same and probably is less than that of the thicker filters.

The filter sheets, received dry, were cut or punched into small pieces of suitable size and shape for the procedures to be described. Following preliminary tests which showed no difference in behavior when spinal cord was placed either on the more shiny or less shiny side of the filter no effort was made to control this orientation. An hour or two prior to use the dry filters were placed for not more than 30 minutes in 70 per cent alcohol, then transferred for a similar period to Tyrode's solution before being stored until use in horse serum—Tyrode's (1:1) solution. While wet, each filter was carefully examined with transmitted light at 18 × magnification and those with significant inhomogeneities were discarded.

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[1] Millipore Filter Corp., Watertown, Mass., U.S.A. Technical information provided in a brochure, "Millipore Filters", distributed by the manufacturer.

Culture methods.—Several methods for incorporating the filter into the cultures were tested. The results to be reported are based on two, which will be designated as the "on-the-clot" and "supported-ring" procedures respectively (Fig. 1). The first is the earlier and more convenient for some purposes, but proved less reliable, particularly with the HA-type filter. The second requires more laborious preparation, but is more reliable and versatile in application. Both are modifications of standard plasma clot culture methods, employing fowl plasma and a nutrient medium of horse serum, Tyrode's solution and 9-day chick embryo juice in 2:2:1 ratio with added penicillin and streptomycin (5 mg of each per 100 ml). In both methods the culture vessel was a round flat "tissue dish"[1], 4.5 cm in diameter and 1 cm high with a central depression or well 2 cm in diameter and of approximately 0.5 ml capacity. The dishes, provided with loosely fitting covers, were incubated on suitable racks in an incubator gassed to maintain approximately 5 per cent CO_2 in air at or close to 100 per cent humidity.

The "on-the-clot" procedure involved placing one tissue on a previously prepared clot in a minimum of fluid, laying a well-drained filter square over the tissue, and then orienting the second tissue over the first in a second clotting mixture. One ml of nutrient fluid subsequently was added as a supernatant which was changed daily. The procedure is simpler but gave less reliable inductions, particularly with the HA filter, than the more laborious but in many ways more convenient "supported-ring" method. The latter represents a modification of methods described by Algire, Weaver, and Prehn [1] for *in vivo* studies with membrane filter "sandwiches".

Discs of filter material (5.5 mm diameter) were cut out of the filter sheet by twisting a small punch against a moderately firm background such as is provided by a blotter on a table top. The discs were cemented to plexiglas (Acrylic plastic, without plasticizer) rings (I. D. = 3 mm; O. D. = 5.7 mm) cut out of 3/32 inch thickness sheets with a drill press. The cement was made by dissolving enough filter material in acetone to yield a fluid of syrupy consistency. A filter disc was placed over a ring and cement carefully applied to the edges with an orally controlled glass pipette of suitable diameter. When the consistency of the cement, which could be varied by adding either acetone or filter material, and the rate of application were proper a smooth seal of the filter to the ring resulted.

Two fine glass rods (ca. 0.2 mm diam., 24 mm in length) were cemented to the plexiglas ring, on the opposite side from the filter, in such fashion as to allow suspension of the ring over the tissue dish well (Fig. 1). Depending upon convenience, the rods were attached to the ring either before or after cementing the filter. The resulting assembly was fairly rugged and could be handled easily with jeweler's forceps without damage to the filter. It provided two culture surfaces, the first within the "cup" formed by the walls of the plexiglas ring, and a second on the opposite side. Cultures could be grown on either or both surfaces, with medium above or below the ring or both.

In preparing spinal cord-metanephrogenic mesenchyme cultures the filter-ring assembly, after passing through 70 per cent alcohol and Tyrode's to horse-serum-Tyrode's, was placed across the well of an empty tissue dish with the cup side up. To the drop of horse serum-Tyrode's carried over in the well a piece of dorsal spinal cord was added. Equal drops of chilled plasma and medium were placed side by side on the shelf of the tissue dish ready to be mixed. The assembly was lifted with jeweler's forceps, touched to the shelf without disturbing the plasma and medium drops, and all excess fluid was removed with a fine pipette (I. D. at tip. ca. 0.2 mm). In the process the nervous tissue was "stranded" near the center of the filter surface, flattened and pressed firmly against it by surface tension. The assembly was returned to its position over the well, the plasma and medium drops combined in the pipette and the resulting clotting mixture added to nearly fill the cup. The spinal cord adhered to the filter and subsequently was supported in this position after the clot had formed.

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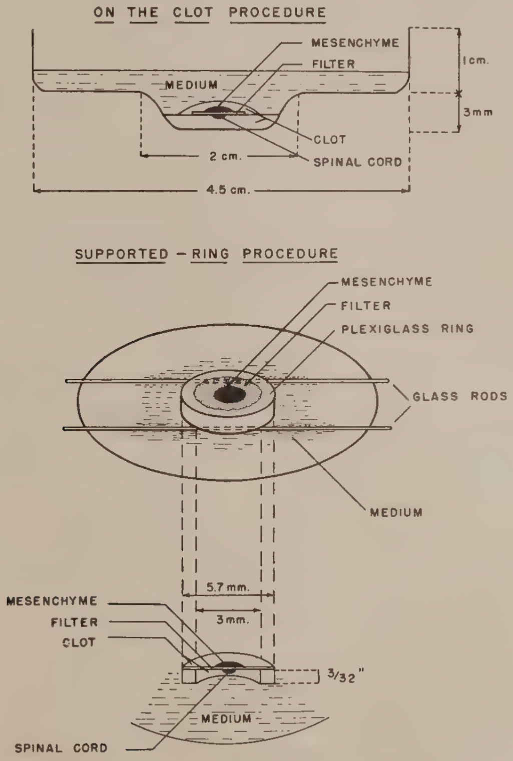
[1] Manufactured by F. Highhouse, glass technologist, whose cooperation is gratefully acknowledged.


ON THE CLOT PROCEDURE
MESENCHYME
FILTER
MEDIUM
1 cm.
3 mm
CLOT
2 cm.
SPINAL CORD
4.5 cm.
SUPPORTED – RING PROCEDURE
MESENCHYME
FILTER
PLEXIGLASS RING
GLASS RODS
MEDIUM
5.7 mm.
3 mm.
MESENCHYME
FILTER
CLOT
3/32"
MEDIUM
SPINAL CORD

Text-fig. 1. Culture procedures. On-the-clot procedure shown in cross-section through entire culture dish. Supported-ring procedure shown in view of tissue dish well above and cross-section of ring below. Diagrammatic, not drawn to scale.

The assembly was then reversed to turn the cup side down. Mesenchyme separated from one metanephric rudiment was deposited over the spinal cord in a minimum of fluid, and excess fluid was again withdrawn. A second clotting mixture, formed as before, was added as a drop on top of the filter. Following full clotting, medium was added first to the cup and then in excess to fill the well. In early experiments enough medium (1 ml) was added to cover the entire preparation, in later ones the amount was reduced to 0.4 ml, enough to nearly fill the well and to provide a "standing drop" in continuous contact with the under side of the assembly. Under the latter conditions the nutrient was "subjacent" rather than "supernatant". In all cases the medium was changed daily.

Tissue source.—Spinal cord and metanephrogenic mesenchyme were obtained from embryos (BALB/C × C3H) on the 11th day after the mother was found with a vaginal plug. General procedures for isolation of the metanephric rudiment and separation of its epithelial and mesenchymal components in trypsin solution have been described [12]. The stage of the rudiments varied from simple ureteric bud to very early branching, with the great majority at the point of flattening and expansion of the bud without recognizable branching. Spinal cord was generally from the cervical and thoracic regions although some anterior lumbar cord may occasionally have been included. The more anterior cord regions were somewhat more convenient, supplying more tissue which was more easily separable from surrounding mesenchyme. For this purpose a short section of cord and adnexa was cut out and turned up on the cut cord surface. A slender iridectomy knife was worked in between spinal ganglia and cord for some distance and then manipulated so as to cut the peripheral tissue away from the dorsal half of the cord. After this was done on both sides, the cord was split in the dorsal mid-line, and the thinner dorsal wall cut in a strip from the thicker ventral wall. In the process any remaining surrounding mesenchyme usually separated without use of trypsin, giving clean dorsal cord. The short strips thus obtained were cut into pieces 0.2–0.4 mm on a side.

Histological.—Cultures routinely were fixed in Zenker-acetic. In the supported-ring procedure the entire assembly was removed from the tissue dish and dropped directly into fixative. After washing, the filter was removed from the ring by gentle dissection around the periphery.

In many cases, particularly with the HA-type filter the spinal cord and its clot would separate from the filter either due to mechanical stresses during removal from the dish or ring, or due to curling of the filter as it went from alcohol to toluene. The latter tendency could be reduced by changing through a close series of increasing toluene concentration. Serial 5 or 10 micron sections, transverse to the plane of the filter, of over a hundred filter-cultures were prepared during the course of the studies. The sections were routinely stained with haematoxylin and eosin which gave the clearest visualization of the filter and contained material. Some cultures were stained with PAS (Hotchkiss) or toluidine blue. The histological observations which are described are representative for the entire series.

All experiments were designed so that comparisons were made between kidney rudiments from opposite sides of the same embryo. Where experiments involved more than two culture types, rudiment pairs were distributed so that each group allowed an equal number of paired comparisons with each other group.

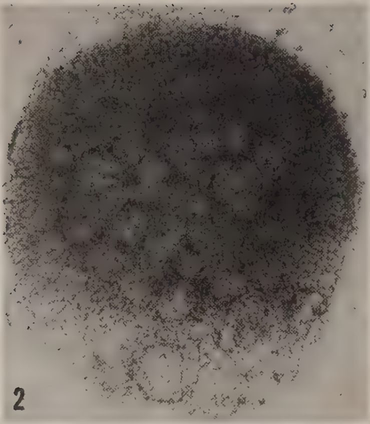
RESULTS

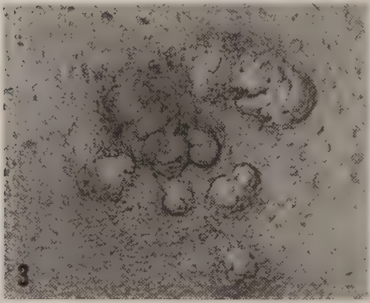
Induction of kidney tubules across the 20A filter.—In one of a number of on-the-clot experiments involving the 20A filter 3 types of culture were prepared: (1) dorsal spinal cord below the filter; (2) dorsal spinal cord below and metanephrogenic mesenchyme above the filter; (3) metanephrogenic mesenchyme above the filter with no spinal cord below. Eight cultures of each type were made, two of each being fixed for sectioning at 20, 30, 40 and 52 hours respectively.

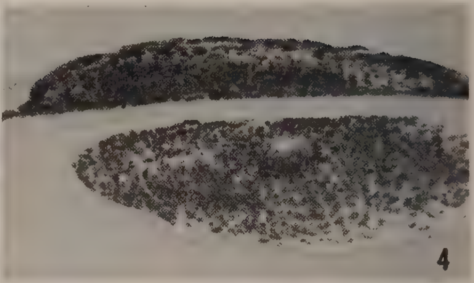
Spinal cord behavior was not significantly different in the presence or absence of mesenchyme. The tissue, up to 52 hours, remained healthy showing little sign of necrosis or degenerative change. The fragment, invariably in close contact with the filter but not always exactly centered under the mesenchyme, frequently reorganized to establish a new lumen (Fig. 4).

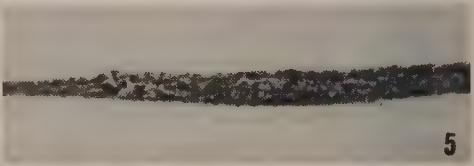
Mesenchymal behavior was quite different in the presence or absence of spinal cord. Without cord the mesenchyme usually flattened rapidly and began spreading over the filter surface very much as on a glass substrate [12]. Although there was some variation, spreading generally had started by 20 hours and was well advanced in all cases by 30 hours (Fig. 5). It continued in later stages, leading to a mesenchymal disc 4–8 cell layers thick which tapered to a peripheral outgrowth sheet a single cell layer thick. The sheet remained cohesive during the 52-hour culture period with no indication of individual cell migration even at the periphery. At 52 hours mitotic activity was still present, though not conspicuous, and the tissue showed no sign of deterioration or degeneration.

The behavior of similar mesenchyme with spinal cord below the filter was distinctly different. Spreading was very much slower, never led to the degree of thinning exhibited by mesenchyme alone, and epithelial tubule rudiments gradually formed in the mesenchyme immediately above the spinal cord. At 20 hours the difference between the two types of behavior was suggested









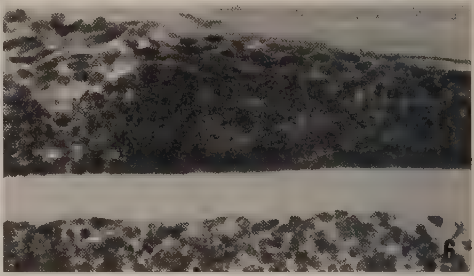


Fig. 2. Tubule rudiments massed over spinal cord with 20A filter between. Near the end of the 3rd day in culture, unfixed. × 60.

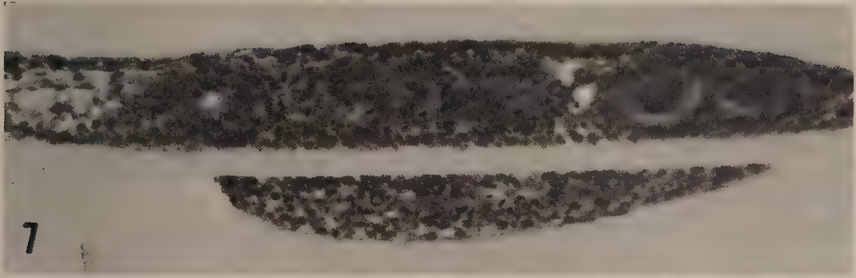
Fig. 3. Tubule rudiments in metanephrogenic mesenchyme on 20H filter after removal of spinal cord from opposite side. Near the end of the 3rd day in culture, unfixed. × 60.

Fig. 4. Metanephrogenic mesenchyme (above) separated from spinal cord (below) by 20A filter. Fixed at 30 hours of incubation. × 160.

Fig. 5. Metanephrogenic mesenchyme on 20A filter with no spinal cord below. Fixed at 30 hours of incubation. × 160.

Fig. 6. Early formation of tubule rudiment in close association with 20A filter with spinal cord below. Fixed at 40 hours of incubation. × 330.

but not yet clear. Observation of the living cultures revealed more consistent spreading of the mesenchyme when there was no spinal cord below, but considerable individual variation was present, particularly in the group with spinal cord. By 30 hours the difference between the two groups was quite clear in the living cultures and was readily apparent in the sectioned material (Figs. 4 and 5). The mesenchymal mass over spinal cord remained



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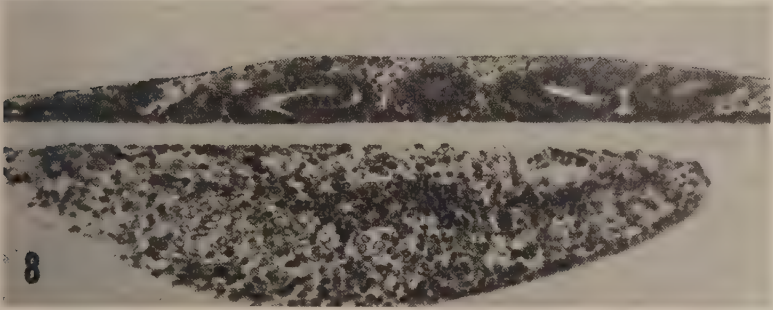

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Fig. 7. Conspicuous cavitated tubule rudiments in metanephrogenic mesenchyme separated from spinal cord by 20A filter. Fixed at 52 hours of incubation. Stained with PAS (Hotchkiss). Section is close to edge of spinal cord, material apparently penetrating filter on mesenchymal side represents debris wiped across upper surface of the section during cutting and partly out of focus. × 220.

Fig. 8. Well-formed epithelial tubules after 65 hours of incubation of metanephrogenic mesenchyme separated from spinal cord by 20A filter. Note that cord tissue now shows signs of deteriorative changes. × 220.

considerably thicker than in the controls. In one of the two cases sectioned the cellular density was greater in the vicinity of the filter and somewhat less above and peripherally (Fig. 4). In the other case the general appearance was similar but the denser area showed local foci of somewhat greater condensation, basophilia and mitotic acitvity suggesting the beginning of tubule rudiments in close association with the filter. When the living cultures had been examined at this stage only one of the six had been recorded as having early tubule rudiments, and this was not one of the two fixed for sectioning.

At 40 hours tubule rudiments were recognizable in three of the four living cultures, and were clearly visible in sections of the two cultures fixed at this time. The rudiments (Fig. 6) were closely associated with the filter surface in the form of local condensations of basophilic cells set off in variable degree from the surrounding looser mesenchyme. They were not as yet well organized, only showing the beginnings of orientation in layers and of change of shape to the columnar form characteristic of tubule epithelium. By 52 hours, however, better organization was present, the forming tubules standing out against the looser mesenchymal background (Fig. 7). The cells in the walls of the tubules were orienting in layers and cavitation of the epithelioid masses was beginning.

Fully organized tubules, and their relationships to the filter, are shown in Fig. 8. The culture illustrated is from another experiment than the one just described and was fixed after 65 hours of incubation. Epithelial character is clear and the tubules in this instance are closely associated with the filter. In other instances at this and later stages the epithelial tubules have been observed to be separated from the filter by mesenchyme. At these later stages, too, tubules were sometimes observed beyond the border of the spinal cord, almost certainly due to peripheral migration in association with the centrifugal mesenchymal spreading.

Comparison of inductive transmission through AA- and HA-type filters.— Early trans-filter inductive effects between spinal cord and metanephrogenic mesenchyme were obtained with HA-type filters using the on-the-clot procedure [10]. These effects were of variable intensity and frequently quite weak or absent. Significantly more reliable results were obtained when the dorsal half of the spinal cord consistently was used as the inductive source, following the finding that this region of the cord is more active than more ventral regions [12]. Still greater reliability was achieved when AA filters were substituted for HA.

In two experiments, for example, a total of 31 cultures of metanephrogenic mesenchyme over dorsal spinal cord were prepared, 16 with 20H filters and 15 with 20A filters. On the 4th day of incubation the mesenchyme in 12 out of 15 20A cultures had formed 15 or more well-developed tubule rudiments, the other three had rudiments which were definite but weakly developed and fewer in number. In the 20H group, however, six cultures were completely negative and none of the other ten had more than five tubule rudiments, all weakly developed. A series of experiments using the 20 A filter and varying a number of procedural details led to the supported-ring technique as a method of greater convenience and reliability than the on-the-clot procedure. With this technique, which assured that the spinal cord was initially firmly adherent to the filter and remained in close contact with it subsequently, consistently positive inductions were obtained with both filter types.

This is indicated in the following experiment in which the supported-ring procedure was used and all known factors were controlled to provide optimum conditions for inductive effects. Twelve cultures were prepared, 6 involving 20A and six 20H filters. At approximately 43 hours of incubation early tubule rudiments definitely were present on all six 20A filters, and on three of the 20H filters. The other three 20H filters were questionable. At approximately 65 hours all cultures showed definite tubule rudiments (Figs. 2 and 3). In the 20A group the number of tubules varied from 14 to 20 with an average of 18.2 tubules per culture. In the 20H group the number varied from 4–12 with an average of 7.0 tubules per culture. Inductive effects, therefore, can consistently take place through the 20H filter but apparently at quantitatively lower levels than through the 20A. Tests of the 30A filter in comparison with the 20A showed no reduction of effect.

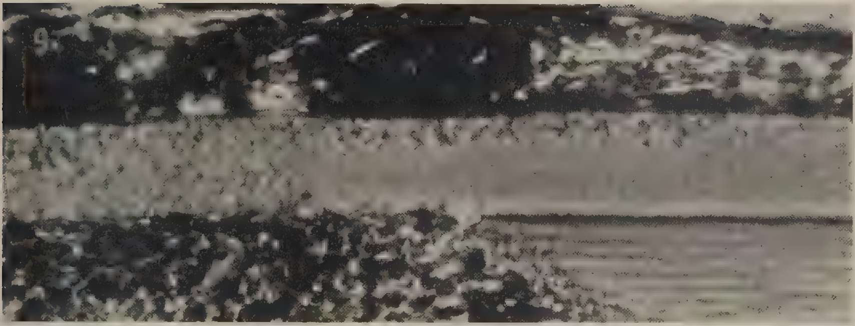
Visual penetrability of the two filter types.—The significance of the observations for distinguishing among the several proposed hypotheses for inductive mechanisms rests on determination of the nature of the materials which cross the interzone. Considerable interest therefore attaches to the question of what can be seen in the body of the filter. In no case has penetration by whole cells been observed. Stainable material penetrating into *AA*-type filters from both sides is, however, readily observable with the highest powers of the ordinary light microscope and stands out still more clearly when phase optics are used. The material includes some lightly staining, faintly granular, pseudopodium-like processes which almost certainly are cytoplasmic extension of cells. But more abundant than these recognizable cytoplasmic processes is heavily staining, flocculent or granular material, the appearance of which, in all of its variations, neither excludes nor demands its interpretation as cytoplasmic extensions of cells. The material is especially conspicuous in the 30A filter (Figs. 9, 10), where the beads or flocculi are quite large and deeply staining, but it is abundant also in the 20A filter (Fig. 11) where the beads are smaller and the impression is of tortuous fibers with local swellings. In both filters, despite much winding and irregular behavior, the overall orientation of the material is across the filter thickness and "fibers" frequently can be traced from one edge well past the middle, or even all the way across. The general appearance is one of interpenetrating projections from both sides with ample opportunity for cross-connection.

The visible material is not limited to the interzone, i.e. the region with tissue on both sides (Fig. 9). Similar penetration is seen where only one tissue is present, whether in sections of cultures involving only one tissue, or in sections of cultures involving both tissues but in regions where mesenchyme has spread peripherally beyond the spinal cord. The material appears to be more abundant and conspicuous, however, in the interzone (Fig. 9).

Penetration of the AA-type filter apparently begins quite early. In one experiment in which cultures were fixed after only 6 hours of incubation numerous fine "fibrous" penetrations were present on the mesenchymal side extending deeply into the filter. Material of more granular consistency was present on the spinal cord side, but not as abundantly or penetrating as deeply. Two cultures fixed at 16 hours showed considerably more penetration from both sides. These preliminary observations may imply that material, optically visible following fixation, is present in the filter as early as 6 hours of incubation but does not reach a maximum until some time later. No firm conclusion can be drawn, however, concerning the possible cytoplasmic nature of the observed material.

Penetrating material is very much less obvious in the HA-type filter (Fig. 12). The general impression with the ordinary light microscope is of almost complete blankness of the filter sections, with only occasional and quite shallow penetration of very fine, slightly beaded fibres. Such penetration has been observed as early as 12 hours of incubation, and on both sides of the filter. With phase optics penetration can be observed to be somewhat more abundant and deeper, but usually does not extend more than 6–8 microns into the filter, i.e. about one-third the filter thickness. However, in a few cultures, particularly among those fixed at 12 hours, fine penetration has been traced deeply enough so that cross-connections between the material from the two sides is conceivable. The fine "fibers" observed grade down in size toward an even finer background fibrillation, just at the limits of phase optics, which is present not only in the region in contact with tissue but in the more peripheral region as well. It appears to reflect the structure of the filter itself.

Deposition of material in the filter by spinal cord.—Penetration of complex materials into both HA- and AA-type filters is further indicated by the following facts. An unfixed piece of spinal cord incubated for approximately 20 hours on the filter in the absence of mesenchyme can readily be lifted off. No visible trace is left in the filter, and no inductive activity is demonstrated if metanephric mesenchyme is cultured over the area of previous spinal cord contact. If, however, the filter with spinal cord in contact is fixed in absolute alcohol-formalin (9:1) for one hour the spinal cord is found to be tightly cemented to the filter, and can be scraped off only with great difficulty. On rehydration the tissue can be removed somewhat more easily, but in either case a "spot" is left in the filter, distinguished from its surroundings



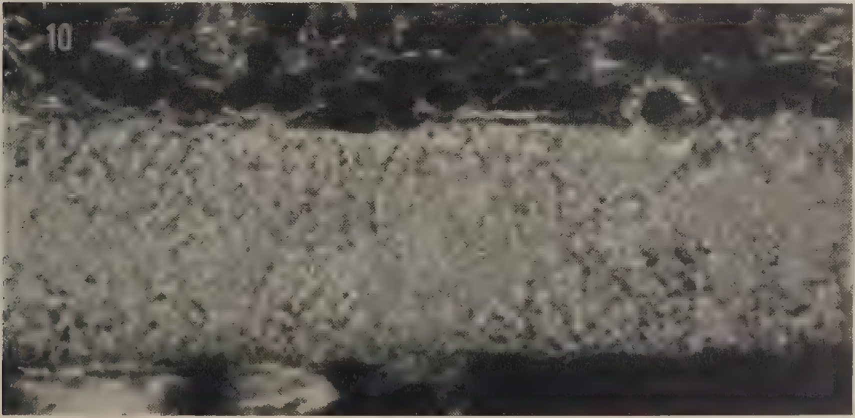
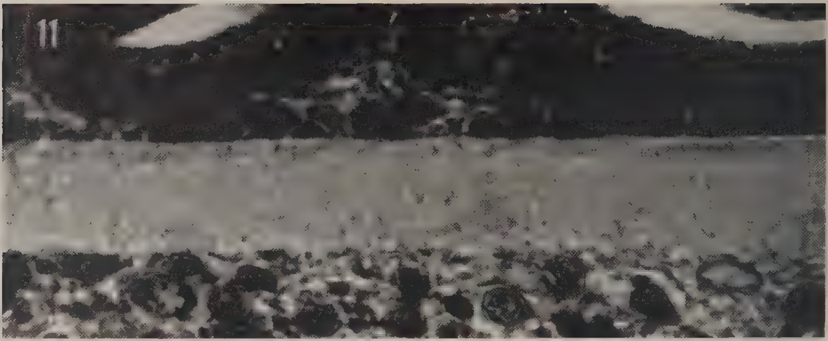


Fig. 9. Penetration of 30A filter as seen in fixed and stained preparation viewed with phase-contrast. Fixed on 4th day of incubation. In photographing, tissues were underexposed in order to bring out detail in lighter filter area. Note that penetration occurs from both mesenchyme above and spinal cord below but is most abundant in "interzone", grading off peripherally. × 530.
Fig. 10. "Interzone" of same preparation as Fig. 9, viewed with 90 × objective under the same conditions. × 1100.

by greater translucency (Fig. 13*A*). The spot is sharply bounded, conforming closely both to the area of adhesion of the spinal cord and to the area of tubule origin if metanephric mesenchyme were present on the upper surface of the filter. If such mesenchyme is placed on fixed and washed spotted filters, however, it spreads as in controls with no sign of tubule formation.

Exposure of spotted filters to trypsin (either crude or twice recrystallized) solution leads to gradual blurring of the outline (Fig. 13*B*), followed by virtually complete disappearance of the spot at 10 minutes of exposure (Fig. 13*C*). The material responsible for the spot appears to shrink during



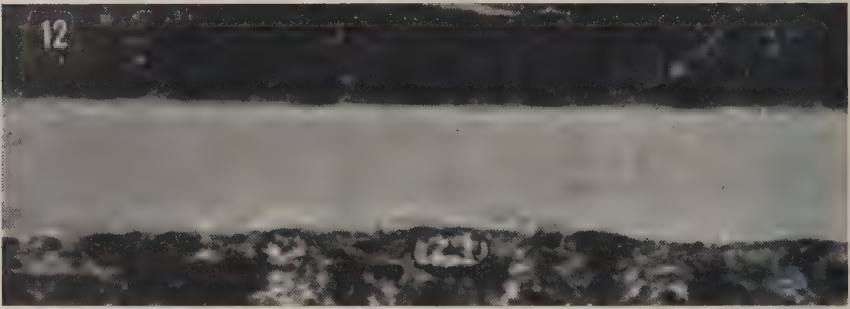


Fig. 11. "Interzone" of culture involving 20A filter viewed as Fig. 10. Fixed on 3rd day of incubation. ×1100.

Fig. 12. "Interzone" of culture involving 20H filter viewed as Fig. 10. Fixed on 2nd day of incubation. Note fine shallow penetration along upper border. The photograph does not bring out all penetration visible by direct observation. ×1100.

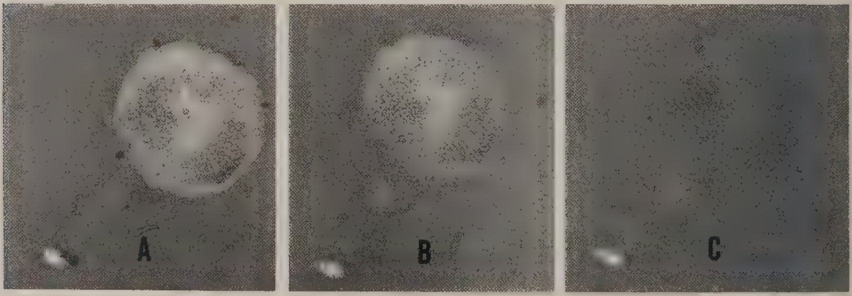


Fig. 13. ×30. *A*. "Spot" left in filter after removal of spinal cord incubated for 20 hours in contact with filter and fixed in alcohol-formalin. *B*. Same "spot" after 3 minutes in 3 per cent trypsin (crude) in Tyrode's without calcium and magnesium salts. *C*. As *B*, after 10 minutes.

fixation in the alcohol-formalin since the spot area is thinned and depressed somewhat with reference to the filter surface to form a small "crater". The spot is refractory to most stains tested. There is no metachromasia and little and variable orthochromatic staining with toluidine blue, similar variability but occasional strong violet staining with the periodic-acid-Schiff (Hotchkiss) procedure and consistent but very light staining with eosin. On the basis of these incomplete data on fixing and staining reactions it seems probable that the spot represents an area where the filter pores have been filled by a protein-rich material of about the same refractive index as the filter. The data are consistent with, but do not require, the interpretation that some of the involved material is glycoprotein in nature.

DISCUSSION

The conclusion may be drawn that the tubule-inducing effect of spinal cord on metanephrogenic mesenchyme can cross an "interzone" up to 30 microns in thickness without passage of whole cells. The effect is strongest with the AA-type filters used, which are penetrated by probable cytoplasmic processes, and by conspicuous granular and flocculent materials of uncertain nature but clearly visible with the optical microscope following fixation. A weaker but regular effect is obtained with lower porosity HA-type filters, in which definite cytoplasmic processes have not been identified, visible penetration being limited to fine beaded "fibers" usually observed to extend only partly across the filter. From the strong localization of tubule initiation immediately over the spinal cord it seems clear that, whatever the nature of the mechanism involved, activity primarily moves perpendicular to the filter surfaces, with little or no tendency to spread peripherally either on the surfaces or within the body of the filter. This behavior is in harmony with the assertion[1] that the filter pore axes predominantly run in this same direction.

From the nature of the procedures required to obtain optimum effects, and consideration of other procedures which lead to technical failures, it seems probable that intimate contact of both tissues with the filter is essential. This recalls the conclusion from amphibian studies that contact of inductive and responding tissues is required in the normal inductive process, a conclusion which is not necessarily negated even if inductive activity can be concentrated in a supernatant solution under the special circumstances recently reported by Niu and Twitty [21]. In the interaction of spinal cord and metanephrogenic mesenchyme it appears, at least, that the required contact need not be between the tissues *en masse*, providing the interzone has certain not yet defined properties which are possessed by the filter.

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[1] See footnote p. 425.

Whether some degree of actual cytoplasmic contact is required cannot be regarded as settled by the reported observations. The presence of abundant optically visible material, some of it almost certainly cytoplasmic, in filters passing maximum activity, and the restricted activity across filters which show much less optically visible penetration, suggests some relationship between the visible material and inductive activity. The situation might be interpreted as favoring inductive dependence upon cytoplasmic contact. There is at the moment, however, no assurance that the bulk of the observed material, particularly the fine beaded fibers seen in the HA-type filter, is not precipitated extra-cellular substance rather than cytoplasm. It is of interest that the appearance recalls the description of processes emitted by amphibian organizer tissue toward overlying epiblast, and the occurrence of fine basophilic filaments in the large pore (3.4 micron) membrane across which Brachet and Hugon de Scœux [5] reported weak induction in the amphibian system. Further study, particularly with the electron microscope, is required before more conclusive statements may be made about the role of cytoplasmic contact in kidney tubule induction. Meanwhile it is worth noting that in relation to inductive mechanisms, too sharp a distinction between cytoplasm *per se* and complex material produced by and immediately contiguous with it may not be of much significance.

The apparent need for contact between tissues and filter, and the strong localization of effects immediately above the spinal cord, seem to militate against the hypothesis of primary involvement of diffusion in tubule induction. The impression is strengthened by the significant restriction of the passage of activity across the 20H filter which, since it does not completely exclude visible penetration as seen in sections, and is permeable to the unidentified proteins of the "spot", would not be expected to exclude most molecules subject to free diffusion. The complexities of transmission through porous membranes [22], however, discourage too rigid a conclusion even on this point.

The available facts, although they do not rule out uncomplicated hypotheses of simple diffusion on the one hand or direct cytoplasmic exchange or interaction on the other, invite consideration of the possible operation of mechanisms in some sense intermediate between the two. The early deposition in the filter by spinal cord of an alcohol-formalin-precipitable and trypsin-digestible material, in a pattern corresponding to the area of tubule initiation, points to such a possible intermediate mechanism.

Inductive mediation by a complex, primarily large-molecular material, organized at the macro-molecular level so as to behave as a cohesive film closely associated with the cells which produce it, would not only rationalize available data on the spinal cord-metanephrogenic mesenchyme interaction, but would fit as well earlier data on epithelio-mesenchymal interaction in the sub-mandibular salivary rudiment [11]. If such an intercellular or matrix material ordinarily were present only in thin films and hence in relatively small quantities, and were soluble and dispersible to ineffective concentrations in physiological fluids, the requirement for intimate association without direct mass cellular contact would be explained. In this event the significant property of the filter would be to collect, and hold in its highly porous structure, surface exudate produced by the tissues and visualized as the "spot" following alcohol-formalin fixation of spinal cord incubated on the filter. It is conceivable that at least part of what is visible in the sectioned AA-type filter, and possibly all in the HA-type, is material of this sort precipitated in the filter channels. In such complex materials, involving simple and probably also conjugated proteins, adequate basis certainly would exist for the specificity of inductive interaction previously reported in *in vitro* inductive systems [11, 12, 14]. The interaction of such complex matrix materials, each produced by and characteristic of the two tissue masses involved, might well lead to alterations of macromolecular organization, possibly along lines suggested by recent studies of fibrogenesis [15, 17]. If the materials were not only produced by the tissues but, as the immediate micro-environment of the cellular components also influenced their character and behavior [7], the alterations resulting from interaction of two dissimilar films might provide the stimulus for the striking cellular reorganization involved in the transformation of massed mesenchyme into epithelial tubules. A speculation of this sort, while not demanded by the facts now available, is in harmony with earlier suggestions of the morphogenetic importance of embryonic ground substance [3, 8, 24] and would serve to link inductive phenomena with emerging understanding of the importance of ground substance and the more highly polymerized components of the matrix in the architecture and function of adult tissues [2, 7, 13].

SUMMARY

1. The tubule-inducing effect of embryonic mouse spinal cord on metanephrogenic mesenchyme can be transmitted across membrane filters of 20–30 microns thickness.

2. The filters completely prevent exchange of cells between the interacting tissues.

3. Across two types of membrane of differing porosity the effects are strongest in the more porous type where considerable penetration into the filter is visible in fixed material, and weaker but definitely present in the less porous type where penetration is at a minimum.

4. The visible material penetrating the more porous filter includes probable cytoplasmic processes, in addition to other material of uncertain nature. The sparse material penetrating the less porous filter does not include any readily identifiable cytoplasmic processes, but the possibility of their existence is not excluded pending further studies.

5. An alcohol-formalin-fixable and trypsin-digestible material is deposited in the filter during incubation in contact with spinal cord. The pattern of its distribution appears to correspond with the area of tubule-initiation when mesenchyme is present.

6. The data are discussed in relation to the nature of inductive mechanisms, and the speculation is advanced that intercellular matrix or ground substance may play a significant role.

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ASTAXANTHIN IN ASTEROID ECHINODERMS *ASTERINA PANCERI*

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It is generally accepted that in animals astaxanthin is formed by the oxidation of carotenoids, probably β-carotene, but the pathway of this oxidation, with the possible intermediates, is still unknown.

Recently de Nicola [1] has suggested, in an asteroid echinoderm, *Ophidiaster ophidianus*, a possible biosynthetic route between some new hydroxyketocarotenoids. In order to check this hypothesis it would therefore seem to be of some value to extend the investigations on astaxanthin to other species of asteroids.

In the shell of *Asterina panceri* some intermediate compounds have been found which are in agreement with the proposed hypothesis of the biosynthetic route of the astaxanthin. The only difference is that the route should be: β-carotene → cryptoxanthin → hydroxyketocarotenoids → astaxanthin; and β-carotene → echinenone → hydroxyketocarotenoids → astaxanthin, in *Ophidiaster ophidianus* and *Asterina panceri*, respectively.

But in any case the hydroxyketocarotenoids are probably the same.

EXPERIMENTAL

Asterina panceri exists in three varieties, green, brown and red. Two hundred animals have been collected in the Bay of Naples and examined. The green, brown and red samples have been studied separately; the analysis showed in the three varieties the same carotenoids in the same relative percentages. The colour is probably due to the linkage between the carotenoids and the protein, since the pigment is present as chromoprotein.

Extraction of the pigment.—The pigments were extracted from the shell of the animals repeatedly with cold acetone. The acetone solutions were concentrated *in vacuo* and the pigment was extracted in diethylether cautiously adding water drop by drop until two layers were formed. The ether solution was washed until free of acetone, dried in Na_2SO_4 and evaporated *in vacuo.* The residue was dissolved in light petrol (35°–55° b.p.). This solution was shaken several times with 90 per cent aqueous methanol (v/v). The methanol solution, containing the hydroxyketocarotenoids (*hypophasic solution*) was extracted with dietylether, as above, in order to obtain a new light petrol solution. The remaining light petrol solution was washed from methanol, dried on Na_2SO_4 and redissolved in light petrol to obtain the *epiphasic solution* (carotenes and ketocarotenoids).

Saponification.—Saponification of the epiphasic fraction was carried out by the method of Goodwin and Srisukh [5].

Chromatography.—The pigments were separated chromatographically using weakened alumina or $CaCO_3$ (B.D.H. Analar) and light petrol (35°–55° b.p.) containing varying amounts of ethyl ether as developer.

Quantitative determination of pigments.—The amounts of pigment were calculated from the extinction value of the different solutions in a known volume of solvent using a Beckman spectrophotometer.

The pigments were dissolved in a known volume of light petrol and the value of the extinction at the point of maximum absorption was taken as measure of the total quantity of pigment. The $E_{1\,\mathrm{cm}}^{1\,\mathrm{per\,cent}}$ (λ max.) values used for known pigments are those given by Karrer and Jucker [6] and Goodwin [3]; for the new pigments the $E_{1\,\mathrm{cm}}^{1\,\mathrm{per\,cent}}$ (λ max.) value has been assumed to be 2500.

RESULTS

The chromatographic separation of the *epiphasic fraction,* after saponification, is given in Table I. Fraction A_1 is β-carotene, A_2 is echinenone, identified by its single banded spectrum, adsorptive power and epiphasic behaviour. Fraction A_3 may be flavochrome from its properties and epiphasic behaviour; fraction A_4 is probably flavoxanthin and is hypophasic. Fraction A_5 is hypophasic and has the same adsorption spectrum as A_4, but much greater adsorptive power; therefore it may be flavoxanthin with further hydroxyl groups. Fraction A_6 is hypophasic and has a single-banded spectrum: it is a ketocarotenoid with hydroxyl groups.

TABLE I

Chromatographic separation of the pigments of the *epiphasic fraction* after saponification. (Adsorbent: weakened alumina; developer: light petrol containing varying amounts of diethyl-ether and/or ethanol; pigments in order of increasing adsorptive power.)

Zone No.	Description	Adsorption maxima (mμ) in light petrol	Conc. of ether in light petrol (v/v) required to elute zone	Identification
A_1	orange-yellow	(420)–448–476	0	β-carotene
A_2	yellow	450	15	echinenone
A_3	yellow	(400)–425–455	30	flavochrome (probably)
A_4	yellow	(400)–425–450	70	flavoxanthin (probably)
A_5	yellow	(400)–425–450	ether + 5 per cent ethanol	hydroxyflavoxanthin (probably)
A_6	red	460	ethanol + acetic acid	hydroxyketocarotenoid

Table II shows the chromatographic separation of the *epiphasic fraction* before saponification.

Fractions B_1, B_5, B_6 are, respectively, β-carotene, echinenone and flavochrome. The other fractions are epiphasic, so it seems correct to consider these as esters, i.e. fraction B_2 the ester of A_6, fraction B_3 the ester of flavoxanthin (A_4) and B_4 the ester of hydroxyflavoxanthin (A_5).

TABLE II

Chromatographic separation of the pigments of the *epiphasic fraction* before saponification. (Adsorbent: weakened alumina; developer: light petrol containing varying amounts of diethylether and/or ethanol; pigments in order of increasing adsorptive power.)

Zone No.	Description	Adsorption maxima (mμ) in light petrol	Conc. of ether in light petrol (v/v) required to elute zone	Identification
B_1	orange-yellow	(420)–448–476	0	β-carotene
B_2	red	460	0	ester of A_6
B_3	yellow	(400)–425–450	3–5	ester of A_4
B_4	yellow	(400)–425–450	10	ester of A_5
B_5	yellow	450	15	echinenone
B_6	yellow	(400)–425–455	30	flavochrome (probably)

TABLE III

Chromatographic separation of the pigments of the *hypophasic fraction*. (Adsorbent: $CaCO_3$; developer: light petrol containing varying amounts of diethylether and/or ethanol; pigments in order of increasing adsorptive power.)

Zone No.	Description	Adsorption maxima (mμ) in light petrol	Conc. of ether in light petrol (v/v) required to elute zone	Identification
C_1	red	—	0	—
C_2	orange-red	470	50	astaxanthin
C_3	red	470	ethanol	astacin

The *hypophasic fraction* has been chromatographed on $CaCO_3$ as described in Table III.

Fractions C_2 and C_3 are the astaxanthin and astacin, respectively. Fraction C_1 was a mixture and was rechromatographed on weak alumina; the results are given in Table IV.

In Table V the relative percentages of carotenoids are given.

Fractions A_6 and D_1 are very similar to the fractions 2F and 2G described in *Ophidiaster ophidianus* [1]. Therefore the same tentative structures still

TABLE IV

Chromatographic separation of the pigments of fraction C_1.
(Adsorbent: weakened alumina; developer: light petrol containing varying amounts of diethyl-ether and/or ethanol; pigments in order of increasing adsorptive power.)

Zone No.	Description	Adsorption maxima (mμ) in light petrol	Conc. of ether in light petrol (v/v) required to elute zone	Identification
D_1	yellow-red	455	60	hydroxyketocarotenoid
D_2	red	460	ethanol × acetic acid	= fraction A_6

TABLE V

The quantitative distribution of carotenoids in the shell of *Asterina panceri.*

Carotenoid	Amount (% of total pigment listed)
β-carotene.	1.0
echinenone	16.0
flavochrome	6.6
flavoxanthin ester	3.5
hydroxyflavoxanthin ester . .	4.5
A_6	12.0
A_6 ester	2.4
D_1	20.0
astaxanthin	34.0

reported [1] of hydroxyketocarotenoids can be assigned to them, which would suggest that they may be intermediates in the synthesis of astaxanthin from β-carotene in *Asterina panceri* also.

DISCUSSION

It is now a well-known fact that astaxanthin, a typical animal carotenoid, is the result of the oxidation of carotenoids taken with the food. It has already been demonstrated in *Locusta* eggs by Goodwin and Srisukh [4] that β-carotene is its precursor.

Recently de Nicola [1], from the study of the pigments of an asteroid echinoderm, *Ophidiaster ophidianus,* has suggested a possible biosynthetic route for astaxanthin, In these animals some new carotenoids have been found, which, owing to their properties, are considered to be hydroxyketocarotenoids. Therefore they have been proposed as intermediate compounds of the metabolic pathway of astaxanthin.

The present work reports the results of a study of the pigment on another asteroid echinoderm, *Asterina panceri;* these seem to fit in with the above scheme for *Ophidiaster ophidiatus,* since similar hydroxyketoderivatives related to astaxanthin are found in this case. Also in *Asterina panceri* astaxanthin is the major component. The scheme for *Ophidiaster ophidianus* (Fig. 1) may be suggested also for *Asterina panceri,* slightly modified (Fig. 2).

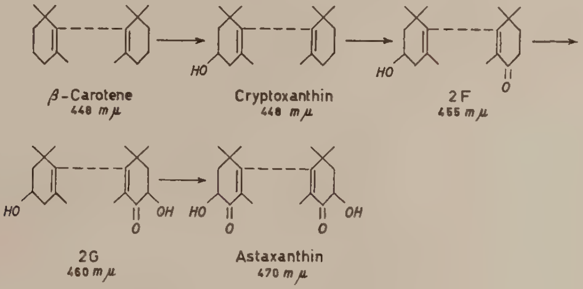
HO
β-Carotene
448 mμ
HO
Cryptoxanthin
448 mμ
HO
O
2 F
455 mμ
HO
OH
O
HO
O
O
OH
2G
460 mμ
Astaxanthin
470 mμ

Fig. 1. Scheme for *Ophidiaster ophidianus.*

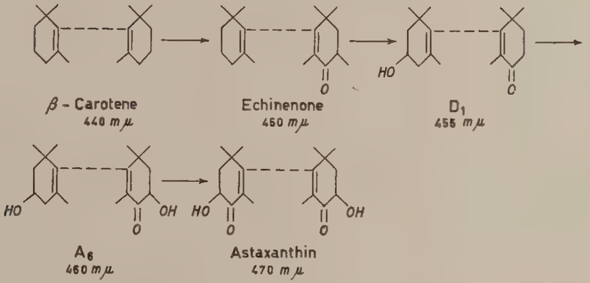
O
HO
O
β - Carotene
440 mμ
Echinenone
450 mμ
D_1
455 mμ
HO
O
OH
HO
O
O
OH
A_6
460 mμ
Astaxanthin
470 mμ

Fig. 2. Scheme for *Asterina panceri.* (Same as for *Ophidiaster ophidianus,* but sligthly modified.)

The only difference would lie in the fact that in *Asterina panceri* the first step of synthesis is echinone instead of cryptoxanthin. This fact is acceptable as the next intermediate compound, i.e. D_1, is probably an hydroxyketoderivative of echinone; it is similar to a carotenoid found in *Paracentrotus lividus* eggs [2], which was considered as above.

These pathways are yet to be demonstrated experimentally, but emphasize the importance of the ketoderivatives in carotenoid metabolism.

In *Asterina panceri* also small quantities of α-carotene derivatives are present, probably flavochrome, flavoxanthin and hydroxyflavoxanthin, which are generally of vegetable origin. *Asterina panceri* feed usually on animals, whose internal organs may contain vegetable residue. The proposed metabolic pathway from the β-carotene to astaxanthin could be ascribed to the presence of a number of enzymes capable of carrying out the oxidation of the β-iononic ring of the carotenoids. Oxidation, in the case of the β-carotene, would then lead to astaxanthin. The presence of α-carotene derivatives may be explained as follows: the possibilities of oxidation of α-carotene are limited to the single β-iononic ring present. Therefore flavochrome and flavoxanthin contain oxygen only in the β-iononic ring. These α-carotene derivatives can be looked on as final products and indeed then are to be found in ester form, i.e. a metabolically less active form.

From a biological point of view it may also be interesting to note that these asteroids have a very different *habitat* though they have an extremely similar pigment metabolism. Therefore it may be inferred that pigment metabolism is much less affected by the surroundings than is usually assumed: it is rather an extremely definite complex of species condition and zoological classification.

SUMMARY

1. The pigments in the shell of the asteroid *Asterina panceri* have been demonstrated and their distribution determined quantitatively.

2. β-Carotene, echinenone, astaxanthin are present, and flavochrome, flavoxanthin and hydroxyflavoxanthin are probably present, in ester form.

3. Two pigments, very similar to those described in *Ophidiaster ophidianus,* are present; these are probably hydroxyketo-derivatives.

4. The possible biosynthetic route from β-carotene to astaxanthin, still proposed for *Ophidiaster ophidianus,* is discussed.

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CONSIDERATIONS OF THE KINETICS OF RESPIRATION

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The respiration carrier chains have been investigated by many workers, e.g. by Chance [1], who has worked out very refined experimental methods. The present paper forms a sequel to a paper of Klein and Runnström [7] where unbranched carrier chains of respiration are treated mathematically. This continuation includes branched chains too. A more complete paper containing the mathematical deduction has been published elsewhere.

The respiration reaction can be described as follows. To oxidize a substance in the metabolism (the substrate) by free oxygen, a number of enzymes, called carriers, are necessary. Cytochrome oxidase (Warburg enzyme), here called carrier number 0, is oxidized by free oxygen. The oxidized cytochrome oxidase is reduced by carrier No. 1. This oxidized carrier is reduced by carrier No. 2. Thus the oxidation is transferred to the substrate, a carrier k being oxidized by carrier $k-1$ and reduced by carrier $k+1$. It is practical to number the chain from the oxygen side, in spite of the fact that most of the oxidations in the chain are transfers of electrons or hydrogen, in other words a transfer from the substrate to the oxygen. The carriers closest to the oxygen can be common to the oxidations of different substrates. After a certain carrier g the chain is branched, because different carrier chains are necessary for the oxidation of different substrates (Fig. 1). The oxidized state of a carrier can also be reduced by the preceding carrier in the chain. This is called a back reaction. In general the rates of these reactions are small in comparison to those of the direct reactions. In the main part of this paper the back reactions are not considered.

The reaction rate in the common chain, i.e. the respiration rate, is denoted R. The branches are numbered 1 to l, and the quantities belonging to the mth branch have a superscript m. Thus the rates in the branches are $\varrho' = \theta' R$, $\varrho'' = \theta'' R$, ... $\varrho^{(l)} = \theta^{(l)} R$. The value of $\theta^{(m)}$ gives the fraction of R that passes the mth branch. Significant for the reaction rate are the reaction velocity constants (λ_k for the oxidation of carrier k by carrier $k-1$) and the total[1] concentrations of the carriers (a_k the total concentration of carrier k). These quantities always appear in the combination $a_{k-1} a_k \lambda_k = R_k$ which number characterizes the corresponding step. Especially $R_0 = \alpha a_0 \lambda_0$, where α is the absorption coefficient of oxygen, a_0 is the total concentration of cytochrome oxidase and λ_0 the reaction velocity constant for the oxidation of cytochrome oxidase by free oxygen. In general the R_k numbers are constant during the reaction, the concentrations of the carriers being constant at unchanged conditions. Assuming s carriers besides cytochrome oxidase, $R^{(m)}_{s+1} = a^{(m)}_s \cdot a^{(m)}_{s+1} \lambda^{(m)}_{s+1}$ is the corresponding quantity for the reaction between the sth carrier and the substrate in the mth branch. $a^{(m)}_{s+1}$ is the concentration of the substrate in the reduced state. In the main part of this paper $a^{(m)}_{s+1}$ is constant as the steady state is considered, but in the application on p. 454 a''_{s+1} varies and thus R''_{s+1} varies.

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[1] The sum of the concentrations of the oxidized and the reduced state of the carrier.

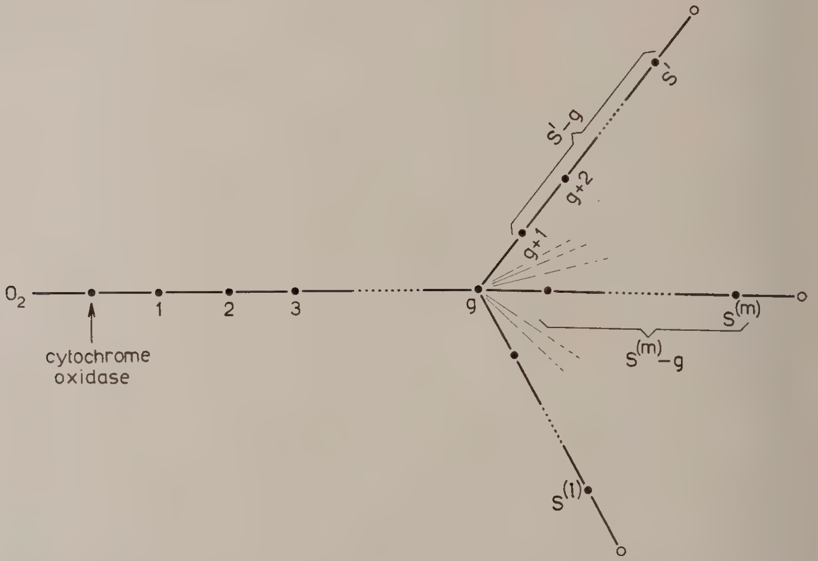

Fig. 1. A figurative chain. The points represent the carriers and the circles the substrates. $s' - g$, $s^{(m)} - g$ and $s^{(l)} - g$ are the numbers of carriers in the branches.

R_n can be interpreted as the maximum velocity that is possible at the corresponding step. This means that $R = R_n$, when all the R_k for which $k \neq n$

are very large in comparison to R_n, so that carrier $k-1$ is approximately kept in the oxidized state by the preceding part of the chain and carrier k is approximately kept in the reduced state by the subsequent chain.

In the general case (Fig. 1) we have an arbitrary number of carriers in the common chain as well as in the branches and an arbitrary number of branches. For this case equations are deduced which connect the oxygen pressure p and the R_k numbers with the reaction rates in the common chain, R, and in the branches, ϱ', ϱ'',... [3]. It would, however, be desirable to express the fraction of R that passes the tth branch, $\theta^{(t)}$, as a function of the R_k numbers in all the branches and of the rate; either the total rate R or the rate $\varrho^{(t)}$ in this branch. Furthermore it would be convenient to express R and ϱ', ϱ'',... explicitly as functions of p and the R_k numbers. This is not possible to do in the general case. Therefore some special cases are treated particularly. Even in these simple cases it is difficult to get a clear view of the relationships, because there are so many constants which are to be determined by experiments in the particular cases. For that reason some numerical examples are given. They are quite hypothetical and are in no way taken from biochemical experiments. The purpose of giving them is to show the character of the formulae in some extreme cases.

RESULTS AND DISCUSSION[1]

i. *Back reactions neglected.*—If there is an arbitrary number of carriers in the common chain but no carriers in the branches, i.e. all the carriers are common to the substrates, then

$$\varrho':\varrho'':\ldots:\varrho^{(l)} = R'_{s+1} : R''_{s+1} : \ldots R^{(l)}_{s+1}. \qquad (1)$$

(No carriers in the branches)

Thus the fractions $\theta^{(m)}$ depend only on the R_k numbers in the branches.

If, on the other hand, there are one or more carriers in the branches, the ratios $\varrho':\varrho''$: ... depend not only on the R_k numbers of the branches but also on the reaction rate. This means that if the branches are unchanged but the common chain or the oxygen pressure is changed, then the fractions $\theta^{(m)}$ are changed. In the special case with an arbitrary number of carriers in the common chain but only one carrier in each branch, the expression of $\varrho':\varrho'':\ldots$ is relatively simple, if it is given as a function of ϱ' instead of as a function of R. Hence

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[1] If according to Green [4, 5] the assumption is made that all the carriers form a large molecule together, the same equations as in [7] and [3] are obtained, differing only in the definition and the values of the λ constants. In this treatment, however, the approximation has been made that the oxidation of one part of the giant molecule by the preceding part is independent of the oxidation state of the rest of the giant molecule.

$$\frac{\varrho'}{\varrho''}=\frac{\theta'}{\theta''}=\frac{\left(\frac{R_s''}{R_{s+1}''}-\frac{R_s'}{R_{s+1}'}\right)\varrho'+R_s'}{R_s''}=\frac{M''\varrho'+R_s'}{R_s''} \tag{2 a}$$

(One carrier in each branch)

If there are only two branches

$$\theta'=\frac{M''\varrho'+R_s'}{M''\varrho'+R_s'+R_s''}, \tag{2 b}$$

where the notation $(R_s''/R_{s+1}'')-(R_s'/R_{s+1}')=M''$ is used.

When ϱ' is very small obviously R_s'/R_s'' determines the distribution between the branches, i.e. the first link in each branch is significant. If ϱ' increases the second step, R_{s+1}' and R_{s+1}'', has more influence. The terms R_s'/R_{s+1}' and R_s''/R_{s+1}'' signify the ratios between the capacities for transmission of the two subsequent steps of oxidation in each branch. When ϱ' increases the ratio θ'/θ'' increases monotonously if $M''>0$, is constant if $M''=0$ and decreases monotonously if $M''<0$, (2 a).

The R_k numbers in the branches may have such values, that one of the branches is dominant (e.g. $\theta'/\theta''\gg 1$) at a low velocity and the other is dominant ($\theta'/\theta''\ll 1$) at a large velocity.

To show how the fractions $\theta^{(m)}$ may vary with the velocity a numerical example is given. It must be pointed out that the numerical values are in no way taken from biochemical experiments. In principle the values are possible, but it is not known, whether they correspond to any state in the living cell.

Numerical example.—There are two branches and one carrier in each. Let

$$R_s'=10^3 \qquad R_s''=10^4$$
$$R_{s+1}'=1.2\cdot 10^3 \quad R_{s+1}''=500.$$

Suppose at first that p and the R_k in the common chain are very large. Then the rates ϱ' and ϱ'' depend only on the R_k numbers in the branches. $\varrho'=R_s'\,R_{s+1}'/(R_s'+R_{s+1}')$ and analogously for ϱ''. Then

$$\varrho'=545,\ \varrho''=476,\ R=1021 \text{ and } \frac{\theta'}{\theta''}=1.14.$$

Suppose now that the common chain is inhibited, so that $\varrho' = 200$. Then

$$\frac{\theta'}{\theta''} = 0.48 \text{ (according to (2 a)),} \quad \varrho'' = 417 \text{ and } R = 617.$$

Suppose finally that the common chain is further inhibited, so that $\varrho' = 100$. Then

$$\frac{\theta'}{\theta''} = 0.29, \quad \varrho'' = 345 \text{ and } R = 445.$$

Thus the ratios between ϱ' in the different cases are

$$545:200:100 = 5.5:2:1,$$

the ratios between ϱ'' in the different cases are

$$476:417:345 = 1.4:1.2:1,$$

the ratios between R in the different cases are

$$1021:617:445 = 2.3:1.4:1$$

and in the different cases θ'/θ'' is 1.14, 0.48 and 0.29.

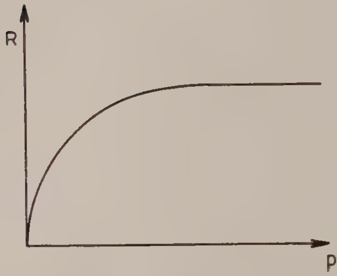
This means that in the uninhibited case ϱ' is 5.5 times larger than in the most inhibited case, but ϱ'' only 1.4 times larger than in the latter case. In the uninhibited case ϱ' is 1.14 times ϱ'', but in the most inhibited case ϱ' is only 0.29 times ϱ''.

At both branched and unbranched chains the respiration velocity is approximately $R = R_0 p$, when p is very small. If the pressure p increases, R increases, and when $p \to \infty$, R asymptotically tends towards a value r_0, see [3] and [7] and Fig. 2. The value of r_0 is generally a function of the R_k numbers except R_0.

If the velocity ϱ' in a branch is plotted against p, a curve of the type of Fig. 2 is also obtained. When p is very small, ϱ' is approximately equal to $\theta_0' p R_0$, where θ_0' is the value of θ', when $R = 0$.

In the treatment of unbranched chains in [7] the case is discussed, where all the R_k for which $k > n$ are very much larger than R_n. Then the oxidation occurs as if the chain ended with the nth carrier. It is limited only by R_n and the preceding part of the chain, and a further increase of these large R_k at the end of the chain, i.e. an increase of the corresponding concentrations, has no influence on the oxidation rate. A comparison may be made with a reaction with a single enzyme, where the reaction constant and the concentration of the enzyme limit the velocity, so that it cannot exceed a certain value, however large the concentration of the substrate may be. The same is valid for branched chains. If all the $R_k^{(m)}$ for which $k > n$ are $\gg R_n^{(m)}$, then a further increase of these large $R_k^{(m)}$ has no influence on the reaction rate in the branch.

It follows from the preceding paragraph that the formulae deduced, when there is only one carrier and a substrate in each branch (see p. 450), are also valid if there are more carriers, provided that only the two carriers in the beginning of each branch limit the rate, and all the following $R_k^{(m)}$ are very large.



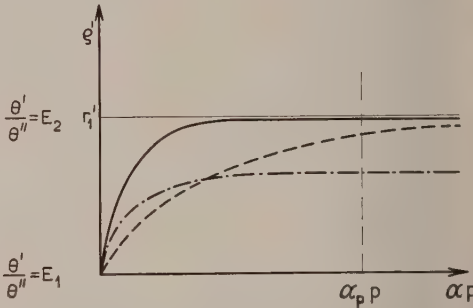


Fig. 2 (left). A schematic curve showing the variation of the respiration velocity R with the O_2 pressure p.

Fig. 3 (right). Schematic curves showing the dependence of the oxidation rate in a branch, ϱ', on the concentration of O_2 in solution, αp, with different values for the R_k numbers in the common chain. $\alpha_p p$ is defined on p. 453. E_1 and E_2 are the extreme values of θ'/θ'', when there are 2 branches and the R_k in the branches are unchanged (cf p. 453 and [3]).

——— R_0/α and the other R_k in the common chain are very large. (Curve 1.)

– – – R_0/α moderate and the other R_k in the common chain very large. (Curve 2.)

—.—. R_0/α very large and at least one R_k in the common chain moderate. (Curve 3.)

The case when two slow steps are separated by a fast step has been treated by Gurney [6].

In Fig. 3 some schematical curves are drawn, which give ϱ' in a branch as a function of αp, the concentration of oxygen in solution. Curve 1 is valid when R_0/α is large. $\theta_0' R_0/\alpha$ gives the slope at $p=0$. In the general case the maximum value of ϱ' depends on all the R_k except R_0. If especially it is assumed that for curve 1 all the R_k in the common chain are very large in comparison to the R_k in the branches, then the maximum value of ϱ' is a function of the R_k' only. If R_0 is decreased a curve of type 2 is obtained. If R_0/α is large but another R_k in the common chain is decreased, a curve of type 3 is obtained. It should be noted that the maximum value of ϱ' is the same for curves 1 and 2, but that it is lower for curve 3. Since the constants in the branches are unchanged in all the three curves, each ϱ' value corresponds to a certain value of θ'/θ'', cf. (2a). On the other hand, if we inhibit in the branch in Fig. 3, when R_0/α and the R_k are large, we obtain a curve of the same type as curve 3, but in this case the ϱ' values do not correspond to the same value of θ'/θ'' as in Fig. 3. The slope of the curve at $p = 0$ is influenced by the changed value of θ_0'.

When there are two branches, one carrier in each, θ'/θ'' increases or decreases monotonously or is constant if the respiration is increased by changes in the common chain and the branches remain unchanged (p. 450). Then θ'/θ'' goes from an extremum E_1 to an extremum E_2, when ϱ' varies from 0 to the largest value that it can reach with unchanged branches. This means, as is seen from Fig. 3, that θ'/θ'' can reach all values between E_1 and E_2 under the conditions corresponding to curves 1 and 2, if p is increased from 0 to ∞, but this is not the case, when curve 3 represents the conditions.

Figs. 2 and 3 can be considered as cell diagrams, showing the rates of oxidation at points at different distances from the periphery of the cell. Some facts can be noticed which are of importance for the regulation of rates. αp is assumed to be the only factor which is unequal in different parts of the cell. This very rough approximation is made to make the properties of the carrier chain evident. It does not invalidate the discussion, because the intention is only to give a schematic survey of the relationships.

Let the value of αp at the periphery of the cell be $\alpha_p p$. Following the actual curve from the point $\alpha_p p$ to the left, we reach a point $\alpha p = \alpha_c p$ which represents the conditions in the centre of the cell. The position of $\alpha_c p$ depends partly on the dimensions of the cell and partly on the diffusion constant. Thus the dimensions of the cell and the diffusion constant may be of great significance for the reactions at the centre of the cell, especially if $\alpha_c p$ lies at the steep part of a curve of type 1 or 3. It should be noted that a large change in ϱ' sometimes corresponds to a large change in θ'/θ'', i.e. a change in the ratio between the branch reaction rates. This fact should be considered in the discussion of cell division processes.

In cells corresponding to curves 1 and 3 a small change in $\alpha_p p$ alters the reaction rates only a little in the outer parts of the cell, but considerably in the inner parts, if $\alpha_c p$ is on the steep part of the curve. On the contrary, in cells corresponding to curve 2, a small change in $\alpha_p p$ alters the rates moderately over the whole cell. If the reaction rates in cells, for which curve 1 is valid, are altered by a change in the carrier chain, so that another curve is obtained, the alterations of the rates are distributed differently in the cell, depending on which of the R_k numbers is changed.

Perhaps it may again be pointed out that each ϱ' value in Fig. 3 corresponds to a certain value of θ'/θ''. This implies that not only ϱ' but also the ratio θ'/θ'' between the rates in the branches is changed, if we move along a curve or from one curve to another.

ii. *Discussion of back reactions.*—In [3] the back reactions are considered only in two special case.The results are as follows.

Even if there are no carriers in the branches, θ'/θ'' depends on the rate besides being dependent on the R_k in the branches. If the rate R in the common chain is below a certain value, one of the substrates is reduced, the other being oxidized ($\theta'/\theta'' < 0$).

If there is one carrier in each branch and only the back reactions between the substrate and the last carrier but not those between the carriers are considered, θ'/θ'' corresponds to (2a), if R'_s is replaced by $R'_s R'_{s+1}/(R'_{s+1} + S'_{s+1})$ and R''_s by $R''_s R''_{s+1}/(R''_{s+1} + S''_{s+1})$. S'_{s+1} and S''_{s+1} stand in the same relation to the back reactions as R'_{s+1} and R''_{s+1} to the forward reactions and thus they are proportional to the concentrations of the oxidized state of the substrates in respective branches.

AN APPLICATION

(2 branches and one carrier in each)

Now a case is considered which illustrates the great significance of the value of θ'/θ'', when the substrates in the two branches are two intermediates in the metabolism of a substance.[1] For instance, succinic acid is oxidized by succinic dehydrogenase and a following step in the metabolism, malic acid, is oxidized by DPN.

We assume that there is a substrate, A', in the branch marked with a dash and that its concentration is kept constant. p and all the R_k except R''_{s+1} are assumed to be constant. The oxidized state of A', denoted A'', is assumed to be further oxidized in the other branch. At first $R''_{s+1} = 0$, but it increases during the experiment. (R''_{s+1} being proportional to the concentration of A'', a''_{s+1}.) When R''_{s+1} is very small, θ'/θ'' is very large, see (2a). As long as $\theta'/\theta'' > 1$, R''_{s+1} will increase. (More A'' is formed than is oxidized. It is assumed, that the same quantity of oxygen is needed for oxidation of one mole of the two substrates.) θ'/θ'', however, cannot be less than the initial value of

$$\frac{-\dfrac{R'_s}{R'_{s+1}}\,\varrho' + R'_s}{R''_s} = T, \tag{3}$$

however much R''_{s+1} may increase, as long as (2a) is valid, since the negative term is limited by the fact that ϱ' cannot increase. (2a) is valid only as long as the reduction of A'' to A' can be neglected. This is the case as long as $S'_{s+1} < R'_{s+1}$, i.e. at least when

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[1] The chain of enzyme reactions of a substance being metabolized as well as the possibility of making an intermediate substance accumulate by inhibition at one stage in the metabolism is discussed by Dixon [2].

$a''_{s+1} \le a'_{s+1}$, if the equilibrium of $A' \to A''$ is far to the right. The type of rection at the steady state depends on the constants in the chains and on the equilibrium constant of the reaction $A' \to A''$. Some typical cases are considered.

(a) $R'_s < R''_s$.

The concentration a''_{s+1} increases and θ'/θ'' decreases. When a''_{s+1} becomes large enough to give θ'/θ'' the value 1, the steady state is obtained, M'' still being > 0 (see (2a)). In this case it is reasonable to neglect the back reaction $A'' \to A'$, because a''_{s+1}/a'_{s+1} is not large, when $M'' > 0$.

(b) T in (3) > 1 at the beginning. Then R'_s must be $> R''_s$.

At first θ'/θ'' decreases rapidly and then more slowly, and A'' is accumulated. Gradually the concentration of A'' becomes so large, that the velocity of $A'' \to A'$ becomes significant for the lowering of θ'/θ'' towards 1. But let us now assume, that A'' can react according to another formula, $A'' \to F$. We assume that this reaction is so slow in comparison to the oxidation, that it can be neglected when the concentration of A'' is moderate, e.g. when $M'' = 0$ in the present case. When more A'' is formed, the reaction rate, f, of the reaction $A'' \to F$ increases at the same time as the rate of $A'' \to A'$. At the steady state $f = \varrho' - \varrho''$, and thus $\theta'/\theta'' > 1$. The relationship between the velocities of $A'' \to F$ and $A'' \to A'$ determines the ratio θ'/θ'' at the steady state, i.e. it determines how much the reaction $A'' \to F$ increases. An example is given below.

(c) The intermediate case, i.e. $R'_s > R''_s$ and T in (3) < 1 at the beginning.

Whether the reactions (c) are similar to (a) or to (b) is dependent largely on the change of ϱ' during the reaction, and this depends not only on the conditions in the branches, but on the R_k numbers of the common chain too.

Numerical examples.—The examples are again hypothetical and the comments on p. 450 are valid here too.

(i) Assumptions: $R'_s = R''_s$. A quantity of A' is added to the carriers, and its concentration is kept constant. The oxidized state, A'', is further oxidized at the other branch and can moreover react according to $A'' \to F$ with the rate f. Furthermore it is assumed that at the steady state $S'_{s+1} = 0.01\ R'_{s+1}$, $f = 0.001\ \varrho'$ and $\varrho'/R'_{s+1} = 0.2$.

By using $\varrho' = \varrho'' + f$ at the steady state we obtain $\theta'/\theta'' = 1/0.999$. If R'_s in (2a) is replaced by $R'_s\, R'_{s+1}/(R'_{s+1} + S'_{s+1})$, (p. 454), it follows that $R''_{s+1}/R'_{s+1} = 0.957$.

(ii) Let us now take another example. We have the same conditions as above, except that R''_s is lowered so that $R''_s = 0.8\ R'_s$, i.e. a''_s is lowered, and (since R''_{s+1} is proportional to a''_s) R''_{s+1} is 0.8 times the value in (i) at the same value of a''_{s+1}. The difference in ϱ' at the steady state between this case and case (i) depends partly on p and on the R_k numbers in the common chain. Suppose that the value of ϱ' lies between $0.16\ R'_{s+1}$ and $0.2\ R'_{s+1}$. That is at least possible to bring about by inhibition in the common chain. The calculations are made under the assumption that the rate f of the reaction $A'' \to F$ is proportional to a''_{s+1} and then

$$f = \frac{R''_{s+1} \times 0.001 \times 0.2 \times R'_{s+1}}{R'_{s+1} \times 0.957 \times 0.8}$$

according to the results in (i). If R'_s in (2a) is replaced by $R'_s R'_{s+1}/(R'_{s+1}+S'_{s+1})$, p. 454, it follows that R''_{s+1}/R'_{s+1} lies between 5.5 and 3.8, the exact value being dependent on ϱ'. This means that the rate of $A'' \rightarrow F$ is between 7.2 and 5.0 times larger than that in [i] but the oxidation rate is only partially changed.

(iii) Let us now take a third example. We have the same conditions in the branches as in (ii) but we assume that it is possible to increase ϱ', so that $\varrho'/R'_{s+1}=0.3$ at the steady state, by changing p or the R_k numbers in the common chain. If we make similar calculations as in (i) and (ii), we obtain $R''_{s+1}/R'_{s+1}=2.0$ at the steady state. This means that the rate of the reaction of $A'' \rightarrow F$ is 2.6 times larger than that in (i).

Summary of results in (i), (ii) and (iii):

If	ϱ'/R'_{s+1}	0.20	0.16	0.20	0.30
and	R''_s/R'_s	1.0	0.8	0.8	0.8

the ratios between the rates f are

$$1 \;:\; 7.2 \;:\; 5.0 \;:\; 2.6.$$

If $A'' \rightarrow F$ in the example is a step in the formation of the carrier corresponding to R''_s or a substance that stimulates the common chain, $A'' \rightarrow F$ may be a regulation reaction.

REMARKS

The carrier chains in the living cell are more complicated in several respects than the chains discussed. They have more carriers and more branches. Furthermore the branches themselves are probably likewise branched, so that there are several junctions. In addition there exist carrier chains for other reactions than oxidation, e.g. phosphorylation. At least the chains of phosphorylation are coupled with the respiration carrier chains. The back reactions ought also to be considered. All these facts make the relationships very complicated. Nevertheless, a schematic calculation such as the one given above can be valuable for obtaining an idea of the properties of the carrier chains and of their significance for the regulation of the reactions.

A characteristic of the chains is that the ratios between the rates in the branches depend on p and on the R_k numbers in the common chain and not only on the R_k in the branches. The examples on p. 455 show how changes in these ratios can be very significant, when the substrates are intermediates in the metabolism of a substance, and thus they show the significance of p and the R_k numbers in the common chain. They show for instance that a decrease of the O_2 pressure may cause an accumulation of an intermediate substance and thus an increase in the reactions $A'' \rightarrow F$. These facts emphasize the significance of the discussion of the rates and conditions at different distances from the periphery (p. 453).

SUMMARY

The kinetics of respiration via carrier chains especially via branched chains are treated. The results can be applied to other reactions of the same type.

In the main part of the paper the back reactions are neglected.

If there are no carriers in the branches and the back reactions are neglected, the ratios between the reaction rates in the branches depend only on the conditions in the branches. If there is at least one carrier in any branch, the ratios between the rates in the branches depend in addition on the reaction rate, so that a change only in the common chain or of the O_2 pressure alters the ratios between the rates in the branches.

The difference in respiration at points at different distances from the periphery of the cell is discussed. The significance of the diffusion constant and the dimensions of the cell is indicated.

The influence of the back reactions is only discussed in some simple cases. Even if there are no carriers in the branches the ratio between the rates of oxidation of the substrates depends on the respiration velocity. If the rate in the common chain is below a certain value the reaction in one of the branches becomes a reduction.

To show the significance of the ratio between the rates in the branches, a case is treated where the substrates are intermediates in the metabolism of a substance.

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UNTERSUCHUNGEN ZUR REPRODUKTION DES TABAKMOSAIKVIRUS

I. ELEKTRONENMIKROSKOPISCHE BEOBACHTUNGEN

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Seit Bawden und Pirie [1] in Extrakten aus Tabakblättern, die mit Tabakmosaikvirus (TMV) infiziert worden waren, nicht infektiöse Fraktionen eines anomalen Proteins fanden und seit Schramm [16] auf chemischem und physikalischen Wege die Aufspaltung der TMV-Partikel in eine Anzahl von definierten Untereinheiten demonstrieren konnte, wurde in verschiedenen Laboratorien intensiv nach dem Mechanismus der TMV-Vermehrung gesucht. Takahashi und Ishii [23], Commoner *et al.* [6], sowie Jeener und Lemoine [10] konnten kurz nacheinander aus Preßsäften TMV-infizierter Pflanzen anomale, nicht infektive and im Vergleich zum TMV niedermolekulare Begleitproteine des Virus isolieren, die sich nach der Methode von Schramm [17] zu Stäbchen normaler Längen reaggregieren liessen. Zech [26] wies das Vorkommen nucleinsäurereicher und nucleinsäurearmer oder nucleinsäurefreier Substanzen mit anderen Methoden an TMV-infizierten Material *in situ* nach. Trotz weiterer Aufklärung der chemischen, physikalischen und sero-biologischen Eigenschaften dieser anomalen Substanzen konnte bisher noch nicht entschieden werden, ob sie Vorläufer, Begleit- oder Abbauprodukte des normalen Virus sind. Sicher scheint nur, dass die relative Grösse des Ribosenucleinsäureanteils und die biologische Aktivität dieser Proteine in unmittelbarem Zusammenhang stehen. Die Frage nach dem Zeitpunkt des Einbaues oder Verlustes der RNS und der damit verbundenen Genäquivalente des TMV ist daher von besonderem Interesse.

Im Gegensatz zu hochpolymerisierter DNS liess sich die TMV-RNS bisher nicht mit Sicherheit elektronenoptisch darstellen. Sigurgeirsson und Stanley [20] fanden nach Alkalibehandlung von TMV-Präparaten und Kanngiesser und Deubner [11] nach Einwirkung verschiedener Chemikalien auf Kartoffel-X-Virus im Elektronenbild dünne Fäden, welche die gesuchte hochpolymerisierte Virus-RNS darstellen konnten. Neuerdings berichten Schramm *et al.* [18], dass intaktes TMV bei pH 10,3 progressiv einen Teil seines Proteins verliert, das dabei in dünne Scheiben vom Durchmesser der TMV-Partikel zerfällt. Im Elektronenbild zeigte sich, dass Stücke von unvollkommen abgebautem TMV-Protein durch einen zentral angeordneten Faden von etwa 34 Å Diameter verbunden sind. Abgespaltene Proteinscheiben haben in der Mitte ein Loch vom Durchmesser des zentralen Fadens. Solche Spaltstücke sind RNS-frei. Die Analyse dieser Fraktion sichert die Identität oder die Verbindung der Virus-RNS mit diesen axialen Fäden. Starlinger [21] hat das RNS-freie Spaltprotein zusammen mit dem X-Protein von Takahashi serologisch untersucht und ihr völlig gleichartiges Verhalten gegenüber Anti-TMV-Serum festgestellt.

Wir haben im Laufe mehrjähriger cytochemischer Untersuchungen über das Schicksal des TMV in der Zelle unter Anwendung der Methodik und der Apparatur von Caspersson [3] die Elektronenmikroskopie zur Kontrolle von Extrakten aus Zellen oder Gewebeteilen bestimmter Infektionsstadien benutzt. Wir sind dabei zu Ergebnissen gekommen, die in einigen Teilen mit denen der genannten Autoren übereinstimmen, in einigen wesentlichen Punkten aber eine andere Deutung zulassen.

MATERIAL UND METHODEN

Tabakmosaikvirus *Marmor tabaci vulgare*, var. *flavum* und die Wirtspflanzen *Nicotiana tabacum*, var. White Burley und *Nicotiana glutinosa*[1] wurden in den Experimenten verwendet.

Infizierung des Pflanzenmaterials.—Zellen von Einzelhaaren wurden individuell infiziert [25]. Ganze Blätter gleichen Alters und vergleichbarer Grössen wurden durch Einreiben der Virussuspension auf Blattober- und -unterseiten mit TMV inokuliert.

Das infizierte Material wurde im Konstantraum bei Dauerbeleuchtung, 25°C und in optimaler Nährlösung gehalten. Das Material wurde nicht nach Zeiten p.i., sondern nach symptomologisch erkennbaren Infektionsstadien der einzelnen Zellen (Phasenkontrastmikroskop) entnommen. Das primär infizierte Material wurde willkürlich in 9 mikroskopisch unterscheidbare Infektionsperioden (IP) [26, 27] eingeteilt.

Extraktion von kontrolliertem Material gleicher Infektionsperioden.—Individuell infizierte Haarzellen wurden in Anlehnung an die vom Carlsberg Laboratorium entwickelten Mikromethoden extrahiert. Die Inhalte von jeweils 100–200 Einzelzellen wurden in einem Medium mit Dichtegradienten von $d = 1{,}089$–$1{,}23$ und 1,28 (Glyzerin- bzw. Saccharosegradienten) isoliert und fraktioniert. Die differentiale Zentrifugierung wurde in der Spinco-Ultrazentrifuge mit Horizontalkopf ausgeführt. Das Verfahren wird ausführlich an anderer Stelle beschrieben.

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[1] Für die freundliche Überlassung des Saatgutes danken wir der Bundesanstalt für Tabakforschung, Forchheim.

Zur Erzielung grösserer Materialausbeute wurde ferner für Zellmaterial der 5.–9. IP ein Standardverfahren angewendet, dessen Auswertung ebenfalls der durchschnittlich extrahierbaren Substanzmenge pro Zelleinheit angepasst wurde. An Stelle des konventionellen Bezugsystems mg Substanz pro g Frischgewicht wurde mg/Anzahl der Zellen pro g Frischgewicht gesetzt. Die in dieser Rechnung eingeschlossene Annahme, dass alle verarbeiteten Zellen der gleichen IP angehören, gleiches Volumen und gleichartige Substanzverteilung ihrer extrahierbaren Inhalte besitzen, führt zwar zu einem beträchtlichen Fehler, der aber nach unseren Erfahrungen weit niedriger liegt als bei Verwendung unkontrolliert nach Zeiten entnommenen Blattmaterials. Das Verfahren ist allerdings erst vom Ende der 5. IP an anwendbar, da sich erst dann grössere Zellbezirke im gleichen oder annähernd gleichen Infektionszustand befinden. Es werden möglichst grosse Teile der oberen Blattepidermis (einschichtige Lage relativ gleichgrosser Zellen) abpräpariert, mikroskopisch untersucht und zusammenhängende Teile gleicher IP entweder kreisförmig ausgestanzt oder rechteckig zugeschnitten. Nach Errechnung der Gesamtfläche dieser Proben Ermittlung der Zellenzahl nach einer Eichkurve, die durch Auszählen von Blattepidermiszellen in einer Zählkammer einmalig aufgestellt wurde. Nach Abwiegen der Proben Extrahierung und Fraktionierung nach der Methode von Takahashi und Ishii [24] mit folgenden Abänderungen: (1) Sämtliche Sedimente und Überstände wurden analysiert und elektronenoptisch untersucht. (2) Die Rohsedimente der ersten beiden Zentrifugierungen wurden 2mal in Chloroform-Puffer pH 7 (1:10) ausgeschüttelt und die wässrigen Phasen zur hochtourigen Zentrifugierung vereinigt. Zur Verminderung des Konzentrationseffektes und der damit zusammenhängenden Verunreinigung mit niedermolekularen Substanzen wurden die Sedimente hochtouriger Zentrifugierungen durch ein- bis zweimaliges Wiederholen des Gesamtprozesses gereinigt. Die erhaltenen Fraktionen wurden bei pH 7 in geeigneter Verdünnung auf die Parlodionfolien zur Elektronenmikroskopie aufgespritzt, zweimal gewaschen und mit Platin-Palladium unter einem Winkel von 25° bedampft. Zentrifuge: Spinco-Winkelzentrifuge; Elektronenmikroskop: RCA.

U.V.-mikrospektrographische Analysen von Proben höherer Konzentrationen wurden im Universalmikrospektrographen nach Caspersson [3] ausgeführt. Untersuchung verdünnter Lösungen der entsprechenden Fraktionen in einem Beckman-Spektrophotometer, Modell DU. Durch das Vorhandensein gefärbter Substanzen in den Niederschlägen wurde die Auswertung der optischen Messergebnisse erschwert. Versuche, diese Stoffe durch Dialysieren oder durch Adsorption an Aktivkohle zu entfernen, führten zu Proteinverlusten. Auf Grund ihres Absorptionsspektrums kann es sich um Pyrrol-haltige Substanzen handeln, die an Protein gebunden sind. Sie wurden hauptsächlich in der sog. Mikrosomenfraktion gefunden.

Das Fehlen geeigneter Bezugsgrössen komplizierte die *quantitative* Koordinierung der stoffspezifischen Absorptionen abzüglich der Tyndall-Streuung in den Lösungen der verschiedenen Fraktionen. Die Raleighsche Beziehung in der allgemeinen Form $\varepsilon = C/\lambda^4$ entspricht als Korrekturformel für die Streuung nicht den Grössenordnungsverhältnissen und der Verteilung der hier besonders interessierenden hochpolymeren Nucleoproteidpartikeln. Die Abhängigkeit des Wellenlängenexponenten von der Partikelgrösse erschwert die Beurteilung der Absorptionsverhältnisse in den verschiedenen Extraktfraktionen, die in sich selbst und im Verhältnis zueinander Teilchen sehr unterschiedlicher Grösse enthalten, deren unspezifische Absorptionen die selektiven überlagern [4]. Die relative Konzentration und die Verteilung der einzelnen Substanzen beeinflussen weiter neben anderen Momenten das Absorptionsspektrum ihrer stoffspezifischen Chromophoren und die Grössenordnung der Streustrahlung. Daher können weder die molaren Extinctionskoeffizienten noch die relative Grösse des Wellenlängenexponenten von einheitlichen Vergleichslösungen bekannter Komponenten als Stoffkonstanten zur quantitativen Auswertung solcher Präparationen herangezogen werden. Weil gerade die zu untersuchenden Virusnucleoproteide im nativen Zustand hohe Konzentration pro Zelleinnheit erreichen können, erschien uns die Verwendung von Modellsystemen konzentrierter Substanz als Bezugsgrösse besonders wichtig.

Als Modell für hochmolekulare und konzentrierte Fasermoleküle ohne Eigenabsorption im selektiven Absorptionsbereich der Nucleoproteide wurden hochgereinigte, proteinfreie Zellwandmembranen unseres Materials (Kontrolle chemisch reinste Baumwolle) in einem Medium von doppelt destilliertem Glyzerin innerhalb des Wellenbereichs von 2600–3500 Å absuchend gemessen. Der ermittelte Wellenlängenexponent war ~2. Als Modell für selektiv absorbierende Virusnucleoproteide untersuchten wir jeweils die gleichen TMV-Kristalle in der Zelle, isoliert und in Lösung. Die Absorptionskurven der intrazellulär gemessenen Kristalle stimmten nach Korrektur der partiellen Absorption des umgebenden Cytoplasmas und der Zellwände mit den Werten für die gleichen herauspräparierten Kristalle gut überein. Diese Messungen ergaben ebenso wie die Daten für aufgetrocknete Filme konzentrierter Lösungen dieser Kristalle (pH 7, 0,05 m Phosphatpuffer) im gleichen Glyzerinmedium einen Wellenlängenexponenten von ~2,3. Verdünnte Lösungen des gleichen Materials zeigten bei neutralem pH im erwarteten Gültigkeitsbereich des Lambert-Beer'schen Gesetzes jedoch starke Schwankungen der Tyndall-Streuung und damit der Grösse der molaren Extinctionskoeffizienten. Im Laufe wiederholter Messungen konnten sich die Wellenlängenexponenten derselben Proben von ~4 nach 3 und niedriger verändern. Auch die umgekehrte Tendenz konnte verfolgt werden. Ähnliche Effekte wurden auch in verdünnten Lösungen reiner TMV-RNS beobachtet.

Einige Gründe für dieses Verhalten können in dieser Mitteilung genannt werden. Bis zur Beendigung eingehender Untersuchungen an Lösungen hoher Konzentrationen und geringster Schichtdicken mit der Linsenküvetten-Methode von Svensson [22] beschränken wir uns auf die Angabe der Quotienten E(265/280) mμ ohne detaillierte quantitative Auswertung unserer Ergebnisse. Die mittleren Extinktionswerte wurden vorläufig für Streuung und Reflexion um den Betrag $-[\varepsilon\, 315(315/\lambda^{2,3})]$ korrigiert.

Infektivitätstest. — Der *Nicotiana glutinosa*-Test wurde nach dem gewöhnlichen Verfahren ausgeführt. Es wurden Gründe für die quantitative Un-sicherheit dieses Testes gefunden. Bawden und Roberts [2] beschrieben den Einfluss kurzfristiger Verdunkelung oder Beschattung der Wirtspflanzen vor der Inokulierung mit TMV auf die Virusvermehrung bzw. die Zahl der Primärläsionen. Aus beschatteten Blättern konnte viel mehr Virus isoliert werden als aus lichtbestrahlten. Schumacher und Halbsguth [19] und neuerdings Lambertz [12] haben das Vorkommen von Aussenwandplasmodesmen und die tages- und jahreszeitlichen Schwankungen ihres Auftretens und Verschwindens eindeutig nachgewiesen. Wir konnten die direkte Korrelation der relativen Rezeptivität von Blättern für Virusinfektion mit der jeweils vorhandenen durchschnittlichen Anzahl von Aussenwandplasmodesmen mit Sicherheit nachweisen. Da der anscheinend notwendige direkte Kontakt von Viruspartikeln mit Organellen des Wandplasmas experimentelle Ansätze zur Klärung des normalen primären Infektionsvorganges zu bieten vermag, sollen unsere Untersuchungen über diese Zusammenhänge anderswo ausführlich beschrieben werden. Für die vorliegenden Versuche wurde der Infektivitätstest stets in den späten Abendstunden ausgeführt (grosse Anzahl von Aussenwandplasmodesmen).

Isolierung der Virus-RNS.—Die Virus-RNS wurde mit der Trichloressigsäuremethode und nach dem Verfahren von Cohen und Stanley [5] isoliert.

ERGEBNISSE UND BEOBACHTUNGEN

Die vergleichende elektronenmikroskopische Untersuchung von fraktioniertem Material aus Zellen der ersten vier Infektionsperioden enttäuschte. Die Grössenordnung der in Dichtegradienten sedimentierten Partikel stimmte weitgehend in gesundem und infizierten Material überein. Abb. 1 a zeigt eine noch nicht gesicherte Ausnahme. Hier sind in und neben einem Kernfragment längliche Teilchen mit einem Durchmesser von etwa 40 Å in grosser Zahl sichtbar, die einem Entstehungszentrum des TMV in der Zelle entstammen könnten. Dieses Präparat wurde aus dem Rohextrakt von Zellen gewonnen, deren pathomorphologischer Zustand in Abb. 1 b wiedergegeben ist: Kernhypertrophie und strahlenförmige Anordnung von cytoplasmatischen Strängen. U.V.-mikrospektrographisch konnten *in situ* in Kern und Cytoplasma anomal hohe Absorptionen lokalisiert werden. Extrakte der 2., 3. und 4. IP zeigten im mittleren U.V. und besonders bei 2600 Å bedeutende Absorptionsunterschiede gegenüber gleich behandelten, nicht infizierten Kontrollen. Die bei der Mikro-Isolierungsmethode anfallenden äusserst geringen Materialmengen und die schwierige Reinigung und Zusammenordnung der Fraktionen lassen bisher noch keine einwandfrei reproduzierbaren Analysen-angaben zu. Es können jedoch mit Sicherheit in der Reihenfolge der frühen IP quantitativ zunehmende anomale Substanzen festgestellt werden, die in der Bande der Nucleinsäuren hoch absorbieren. Sie sind im Extrakt wahrscheinlich niedermolekular, wenig stabil und mit Bestimmtheit nicht infektiv.

Mit Beginn der 5. IP (X-body-Bildung) treten plötzlich und ohne wahrnehmbaren Übergang grosse Mengen der bekannten TMV-Partikel im Elektronenbild auf. Bei früheren Isolierungsversuchen gingen wir vom Frischgewicht infizierter Blattstücke aus, die mikroskopisch so weit kontrolliert waren, dass mit einer Mehrzahl von Zellen der gewünschten IP zu rechnen war, in denen aber sicher viele Zellen früheren IP angehörten oder noch nicht infiziert waren. Hier ergab sich pro g Frischgewicht eine bedeutende Zunahme extrahierbarer Virussubstanz von der 5. bis zur 8. IP. Legten wir aber dem Frischgewicht als Bezugsgrösse die Anzahl kontrollierter Zellen einer bestimmten IP zu Grunde, kamen wir zu dem unerwarteten Ergebnis, dass die isolierbare Menge Virussubstanz pro Zelleinheit keine statistisch signifikante Zunahme während der 5.–8. IP aufwies. Die mittlere Fehlerbreite der Einzellenmethoden ist zwar noch beträchtlich (15–25 %), da bisher die Zellvolumina noch nicht berücksichtigt wurden, die Isolierungsprozeduren untereinander mit Unsicherheiten behaftet sind und da wohl auch mit verschiedenen physiologischen Reaktionen verschiedener Zellen zu rechnen ist. Trotzdem spricht aber dieses Resultat deutlich für die Annahme der *Entstehung der gesamten Virusnachkommenschaft in einem Wurf pro Zelleinheit*. Dieser Befund bildet die Grundlage für die Beurteilung aller weiteren Untersuchungen über das Schicksal der infizierenden TMV-Partikel.

Im Elektronenbild waren in Extrakten der 5., 6. und auch noch der 7. IP vorwiegend lange TMV-Stäbchen nachweisbar (Abb. 2). Es fiel aber auf, dass in frisch verarbeiteten Präparaten in der Reihenfolge der IP proportional mit der allmählichen Abnahme langer Partikel zunehmend Bruchstücke verschiedenster Grössenordnungen auftraten, die sich sämtlich durch pH-Erniedrigung des Ausgangsextraktes zu langen, morphologisch vom TMV nicht unterscheidbaren Partikeln reaggregieren liessen. Aus Präparaten der 9. IP konnten mit Leichtigkeit grosse Mengen ± isodiametrischer Teilchen isoliert werden, die wohl mit dem X-Protein von Takahashi identisch sein dürften (Abb. 3). Die Absorptionsverhältnisse solcher Extrakte zeigen mit Zunahme an Bruchstücken die Abnahme des RNS-Gehaltes an. Die Schwankungen im RNS-Gehalt wurden im Beckman-Spektrophotometer gegen einen Standard von Virusprotein + ~6% RNS in 0,1% Lösungen weitgehend gereinigter Extrakte abgelesen. Die Korrelation des relativen RNS-Gehaltes der Präparate mit der Partikellänge kommt in diesen vergleichenden Untersuchungen klar zum Ausdruck. Die Abnahme der RNS-Absorption mit zunehmendem Infektionsalter liess sich gleichfalls durch Messungen an intakten Zellen entsprechender IP demonstrieren [27].

Tabelle I gibt ein Übersicht über die Verteilung der Partikellängen, der Absorptionsverhältnisse und der relativen biologischen Aktivität von Extrakt-Fraktionen der 5.–9. IP.

Tabelle I. In der ersten Längsspalte sind die Sedimentproben von vier hochtourigen Zentrifugierungen (140000 × *g*) der Ausgangsextrakte der in der Horizontalspalte aufgeführten einzelnen IP angegeben, die verhältnismässig substanzeinheitliches Material ergaben. Die einzelnen IP sind in je 5 Längsspalten unterteilt. Die 3 ersten enthalten Angaben über die abgeschätzte Längenverteilung der Partikel innerhalb einzelner Proben. Es erschien uns ausreichend, die Einteilung nach langen Partikeln (Normallängen und länger), mittleren Partikeln (bis herab zu 1/3 der Normallänge) und kurzen Partikeln (bis zu isodiametrischen Teilchen) vorzunehmen. Sehr kurze, schwer identifizierbare Teilchen wurden vernachlässigt. Es handelt sich also bei diesen Angaben nicht um absolute Werte.

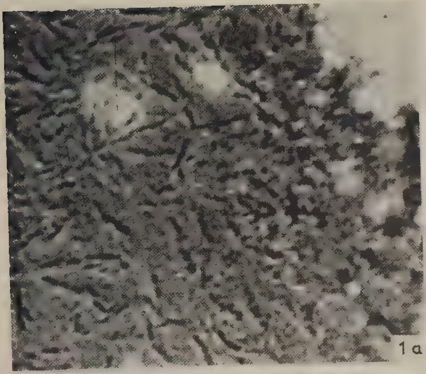
In der 4. Längsspalte unter jeder IP sind die Ergebnisse der U.V.-spektrographischen Messungen für die einzelnen Proben angegeben, und zwar als die um die Lichtstreuung korrigierten Quotienten der Extinktionen bei 2650 und 2800 Å. Die 5. Längsspalte pro IP enthält die übliche Kennzeichnung für die relative biologische Aktivität der Proben im *N. glutinosa*-Test.

++++ hoch infektiv; +++0 infektiv; ++00 schwach infektiv; +000 kaum infektiv; 0000 nicht infektiv.

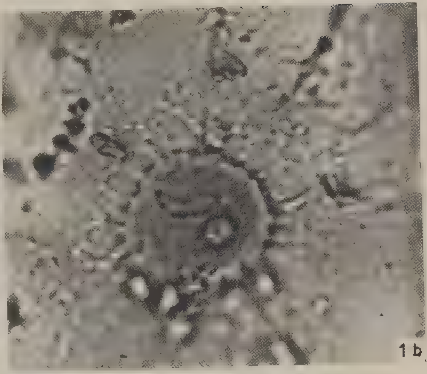
Sämtliche Daten sind aus den Mittelwerten von 3 verschiedenen Präparationen der gleichen IP und aus je 5 Einzelbestimmungen pro Probe gewonnen.

Die relative Höhe der QE in den verschiedenen Fraktionen der IP-Reihe wirft die Frage nach dem realen RNS-Gehalt infektiver und intakter TMV-Teilchen auf. Schon Bawden und Pirie [2] fanden in jedem Extrakt aus TMV-infizierten Blättern neben aktivem Virus einen wechselnden Anteil nicht infektiver TMV-Substanz. Aus unseren Versuchen geht hervor, dass das Infektionsalter der Zellen und der jeweilig extrahierbare RNS-Anteil des TMV-Nucleoproteids in Zusammenhang stehen. Hochgereinigte Präparate der 7. IP (1. Kristallstadium) enthielten bei einem QE von ~1,0 nach unseren Ermittlungen etwa 5,6% RNS. Ein solches hoch infektives Präparat dürfte den Proben entsprechen, die allgemein zur RNS-Analyse benutzt wurden.

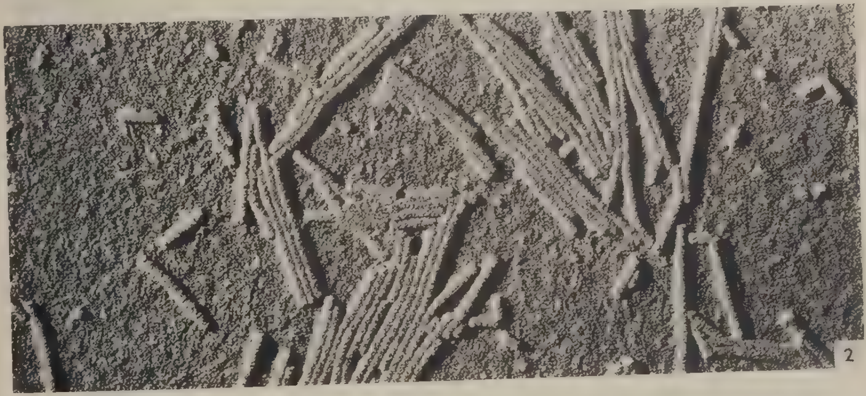
Abb. 1a. Kernfragment oder Cytoplasma mit zahlreichen fädigen Partikeln. 2. IP. ×85000.
Abb. 1b. Hypertrophierter Kern einer Zelle der 2. IP mit typischer Anordnung der cytoplasmatischen Stränge. Die lichtbrechenden Körper unter dem Kern sind Plastiden ausser Focus. Fix. Regaud. Pyronin-Methylgrün. ×1000.
Abb. 2. TMV-Fraktion aus Zellen der 6. IP. ×85000.
Abb. 3. X-Protein aus Zellen der 9. IP. ×85000.



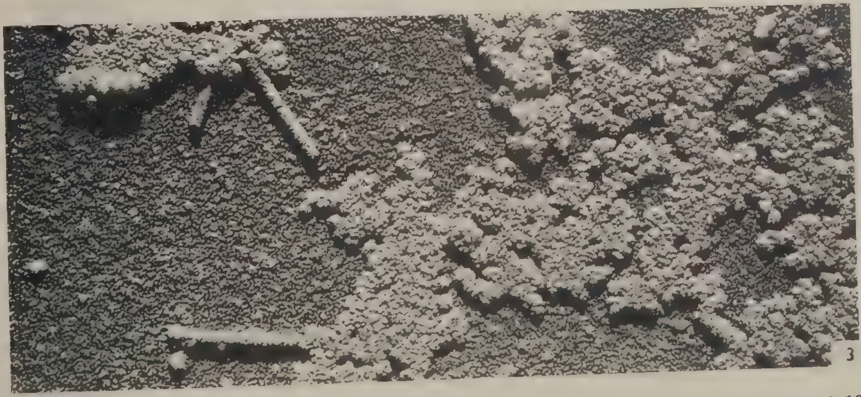
1 a



1 b



2



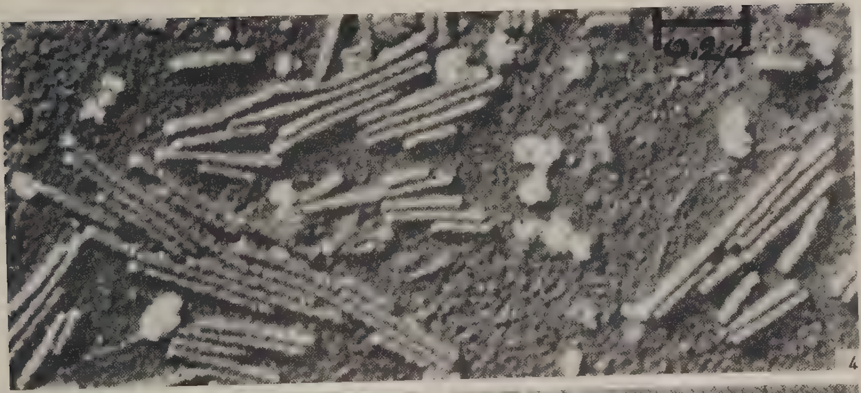
3

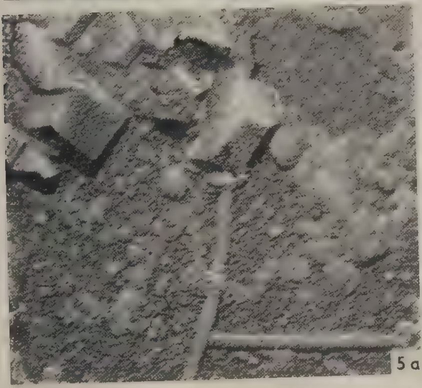
TABELLE I

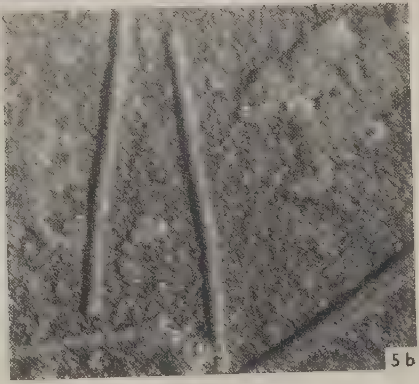
Reihenfolge der hochtourigen Zentrifugierung.	5. IP (X-body-Bildung)					6. IP (X-body-Stadium)					7. IP (1. Kristallbildg.)					8. IP (2. Kristallbildg.)					9. IP (Fibrillenstadium)				
Sedimente pH 7	l	m	k	QE	I	l	m	k	QE	I	l	m	k	QE	I	l	m	k	QE	I	l	m	k	QE	I
1. UZ	85	10	5	1,3	+ + + +	80	10	10	1,4	+ + + +	80	30	15	1,1	+ + + +	20	65	15	0,84	+ + 00	5	5	90	0,63	+ 0 00
2. UZ	2	80	18	1,25	+ + + +	10	70	20	1,2	+ + + +	10	45	55	0,96	+ + + 0	–	15	85	0,70	+ 0 00	–	5	95	0,54	00 00
3. UZ	–	100	–	1,25	+ + + +	–	80	20	1,07	+ + + +	–	10	90	0,78	+ + 00	–	–	100	0,19	00 00		–	100	0,29	00 00
4. UZ	–	–	–	0,37	00 00	–	40	60	0,59	+ + + 0	–	5	95	0,81	+ 0 00	–	10	90	0,56	00 00	–	–	100	0,21	00 00

UZ = Ultrazentrifugierungen
l = lange Partikel %
m = mittlere Partikel %
k = kurze Partikel %

$QE = QE \frac{2650}{2800}$ Å
I = Infektivitätstest







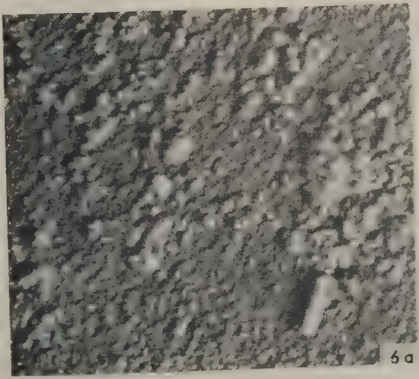
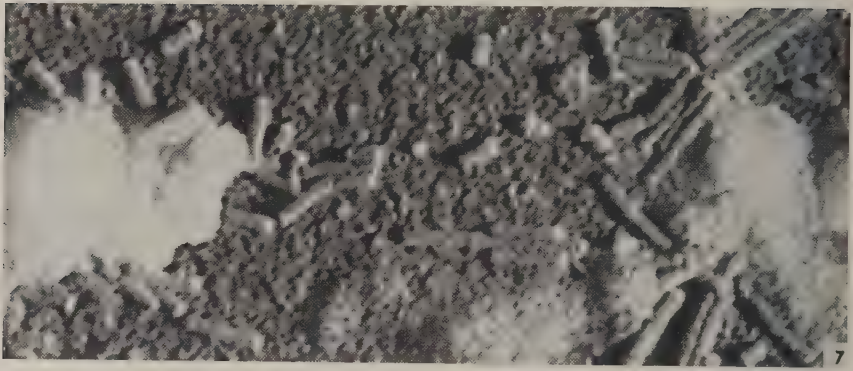


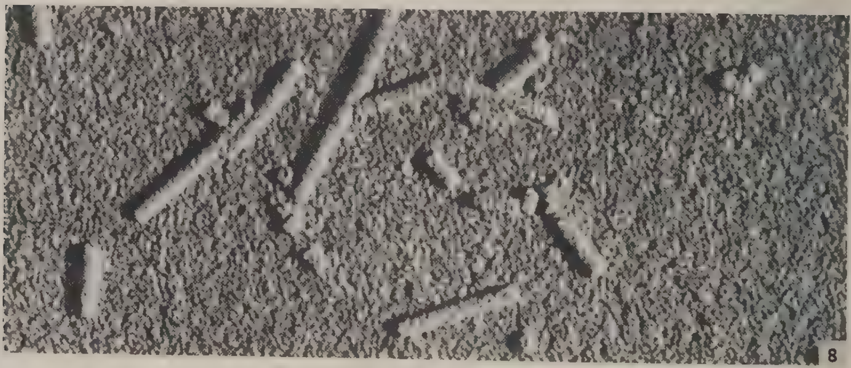
Abb. 4. TMV-Partikel und ausgestossene Zentralfäden. pH der Lösung = 7,0. × 85 000.

Abb. 5a und 5b. ± Salzfreies Präparat der 5. IP. 1. Ultrazentrifugierung, pH 5,6. Längsaggregierte TMV-Partikel, Kristalle und präzipitiertes Protein. × 85000.

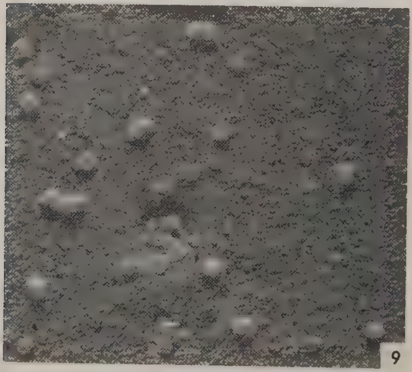
Abb. 6a und 6b. Die gleiche Substanz bei pH 7. Die vorher zahlreichen Viruspartikel sind zum grossen Teil zerfallen. Abb. 6b zeigt rechts verschiedene Zerfallsstadien. × 85000.



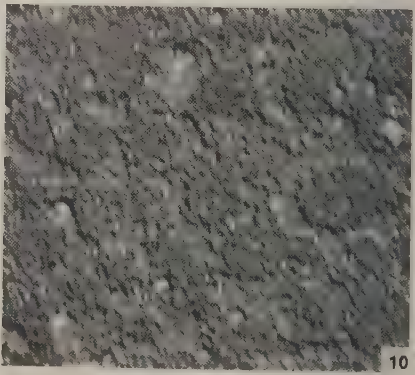
7



8



9



10

Messungen an Substanzen der 5. und 6. IP ergaben aber höhere QE-Werte. Da der Tyndall-korrigierte QE von reiner 0,1 % TMV-RNS schon bei 1,92 lag, ist es wahrscheinlich, dass der reale RNS-Gehalt intakter infektiver Partikel höher ist als 6 %.

Die im experimentellen Teil angeführten Schwankungen der Extinktionskoeffizienten von verdünnten Lösungen unseres Materials dürften zum Teil auf die Labilität vieler Partikel zurückzuführen sein, die zu wechselnden Formveränderungen im Lauf der Messungen führt (siehe unten). Die Instabilität der TMV-RNS ist bekannt.

Über die Zulässigkeit vergleichender intra- und extrazellulärer cytochemischer Messungen gibt Tabelle II Auskunft.

Tabelle II.—In dieser Tabelle sind U.V.-mikrospektrographische Modellmessungen an Viruskristallen der 6. and der 7. IP dargestellt. Die Zahlen geben die Quotienten E (2650/2800) Å korrigiert für Lichtstreuung und Reflexion wieder. Spalte I: Kristalle, intrazellulär gemessen. Spalte II: Die gleichen Kristalle extrazellulär gemessen. Spalte III: Messungen an 3–5 μ dicken Filmen von gelösten Kristallen entsprechender IP. Medium in allen Fällen doppelt dest. Glyzerin. Absuchende Messungen, durch jedes Objekt wurden 10 Meßstrecken gelegt. Gemessene Fläche pro Objekt $\sim 6\,\mu^2$. Es wurden die Mittelwerte der Integrale der gemessenen Absuchstrecken verwendet.

Objektiv: 2,5 mm Quarzmonochromat, numerische Apertur 0,85. Glyzerinimmersion $n = 1{,}445$, korrigiert für $\lambda = 2750$ Å. Quarzkondensor, numerische Apertur etwa 0,3; Glyzerinimmersion $n = 1{,}473$. Quarzokular 5 ×.

TABELLE II

	I QE $\frac{2650}{2800}$ Å	II QE $\frac{2650}{2800}$ Å	III QE $\frac{2650}{2800}$ Å
7. IP	1,05 ±0,02	1,08 ±0,004	1,1 ±0,05
8. IP	0,80 ±0,02	0,82 ±0,005	0,80 ±0,04

Abb. 7. Zerfallende TMV-Partikel in Kontakt mit Substanz aus Rohextrakt. pH 7. ×130000.

Abb. 8. Hochgereinigtes und ursprünglich sehr infektives Präparat der 7. IP, das bei pH 7 und 1°C mit Rohextrakt 1:50 versetzt wurde. Fraktion 1: Partikel normaler Längen und grössere Bruchstücke. ×130000.

Abb. 9. Fraktion 2 desselben Präparates: kurze Partikel und Scheiben verschiedener Dicken mit zentralem Loch. ×130000.

Abb. 10. Fraktion 3: Fortgeschrittener Zerfall des TMV bis unter die Grössenordnung des „X-Proteins". ×130000.

Aus der Tabelle geht klar hervor, dass die messbare Verschiebung des Virus-RNS/Virusprotein-Verhältnisses innerhalb unterscheidbarer IP in der Zelle, an intakter isolierter Virussubstanz und in Lösungen der gleichen Substanz übereinstimmt.

In Elektronenbildern von Präparaten, die nicht sehr hoch gereinigt waren und die meistens der 7. IP (1. Kristallbildung) angehörten, beobachteten wir immer wieder fädige Gebilde in unmittelbarer Nähe der TMV-Stäbchen (Abb. 4). Sigurgeirsson und Stanley [20] fanden solche Fäden in alkalischen Hydrolysaten des TMV. Es konnte sich hier um die hochpolymerisierte Virus-RNS handeln. Obwohl unsere Präparationen sämtlich bei neutralem pH auf die EM-Objektträger aufgebracht und die getrockneten Filme mit Aq. dest. gewaschen worden waren, erschien es denkbar, dass örtlich stärkere pH-Verschiebungen vorkamen und so eine Aufspaltung der TMV-Partikel veranlassten. Es wurden darum Sedimente in Aq. dest. aufgenommen, mit Aq. dest. weiter verdünnt und die so ziemlich salzfreien Lösungen mit einem End-pH von 5,6 auf die Parlodion-Membranen aufgespritzt. Die Abb. 5a und 5b zeigen ein solches Präparat der 5. IP (Anfänge der X-body-Bildung), und zwar aus dem Sediment der 1. Ultrazentrifugierung. Neben längs aggregierten TMV-Partikeln sind diffuse Proteinmassen und gut ausgebildete kubische und hexagonale Ultramikrokristalle sichtbar. Zum Vergleich wurde eine Probe der gleichen Herkunft auf pH 7,0 eingestellt, und es zeigte sich elektronenoptisch das überraschende Bild des Zerfalls der meisten vorher sichtbaren Strukturen in kleinere Partikel, einschliesslich des Hauptanteils der TMV-Stäbchen (Abb. 6a und 6b). Dieses Phänomen führte zu der Überlegung, dass in der auf Abb. 5a sichtbaren non-Virussubstanz ein Stoff vorhanden sein müsste, der im neutralen oder schwach alkalischen Medium den Zerfall des Hauptanteils der TMV-Partikel auszulösen vermag. Der Vorgang konnte mit anderem Material reproduziert werden. Es kann sich um den Inaktivierungsstoff handeln, den Melchers und Schramm [13] in Blattpreßsäften festgestellt haben. Da wir trotz jahrelangen Suchens in Extrakten frisch infizierter Zellen und Gewebe niemals die Spur einer intakten TMV-Partikel feststellen konnten, lag es nahe, den Zerfallseffekt direkt mit dem Infektionsmechanismus des TMV in Zusammenhang zu bringen.

Wir stellten folgenden Versuch an: Einem hochgereinigten Viruspräparat mit vorwiegend langen Teilchen wurden Spuren von Rohextrakt auf dem EM-Objektträger zugesetzt. Abb. 7 demonstriert den beginnenden Verfall der Partikel, die im Kontakt mit der Rohsubstanz oder in ihrer unmittelbaren Nähe lagen.

TMV-Teilchen in hochgereinigten Lösungen neigen eher zur Aggregation als zum Zerfall, wenn sie längere Zeit bei 1°C aufbewahrt werden. Nach längerer Einwirkung von geringen Mengen der Rohsubstanz auf ein solches Präparat *in vitro* liessen sich ohne Mühe folgende Fraktionen mit der Ultrazentrifuge isolieren: Eine Fraktion mit normalen Partikeln und vielen längeren Bruchstücken (Abb. 8). Eine andere Fraktion mit Bruchstücken kurzer und kürzester Längen und vielen sehr dünnen Scheiben, die sämtlich zentral durchlöchert sind (Abb. 9). Bei genauerer Beobachtung des Präparates lassen sich weiter noch sehr dünne Partikel von geringerem Durchmesser erkennen, deren Lochgrösse aber unverändert erscheint. Andere beginnen vollständig zu zerfallen. An den Polenden vieler TMV-Bruchstücke fallen optisch dichtere Stellen auf, die oft entgegengesetzt zur Beschattungsrichtung liegen und den Eindruck einer anhaftenden Substanz erwecken können. Abb. 10 zeigt die nächste Fraktion in einem fortgeschritteneren Zerfallsstadium von netzartig aufgetrockneten minimalen Teilchen und dazwischen liegenden optisch sehr dichten Partikeln, die mit den genannten Verdichtungen der Polenden identisch sein können.

Aus diesem Versuch geht hervor, dass die TMV-Partikeln bei neutralem pH im Laufe des Isolierungsprozesses in kleinste Einheiten bis zum X-Protein und darüber hinaus zerfallen können.

DISKUSSION

Commoner und Rodenberg [7] konnten RNS-freies anomales Protein erst nachweisen, wenn das TMV etwa 1/3 seiner maximalen Konzentration im infizierten Blattstück erreicht hatte, Delwiche *et al.* fanden N^{15} erst 144 h p.i. im X-Protein wieder. Beide Arbeitsgruppen sehen deshalb das anomale Begleitprotein der Virusinfektion nicht als direkten Vorläufer des TMV an. Die Isotopenversuche dieser Verfasser und auch die von Jeener [10] zeigen den sehr lebhaften Einbau von N^{15} in die jeweilig gefundenen anomalen Proteine, der der Radioaktivität in der eigentlichen TMV-Fraktion ungefähr entspricht. Meneghini und Delwiche [14] fanden bis zum 5. d p.i. viermal so viel N^{15} im TMV wie in den Zellproteinen. Darauf fällt der Einbau der Isotop-Ionen in das Virus stark ab, während er bei den Zellproteinen konstant bleibt. 30 d p.i. wird in primär infizierten Blättern praktisch kein Isotop mehr in das TMV-Nucleotid eingebaut. Nach Schönfellinger und Broda [15] wird assimilierter C^{14} ausschliesslich in die Virusnucleinsäure eingebaut, und die spezifische Aktivität des Isotops im neu synthetisierten Virus entspricht in der ersten Zeit p.i. der spezifischen Aktivität des gesamten Blatt-Kohlenstoffs.

Keines dieser wertvollen Ergebnisse vermag einzeln oder in Ergänzung der anderen etwas über die Natur und das faktisch erste Auftreten der anomalen Begleitproteine des TMV auszusagen. Allen diesen Versuchsanordnungen ist gemeinsam, dass infiziertes Blattmaterial nach Zeiten geerntet und extrahiert wurde und dass somit die Proben der frühesten Infektionsstadien vorwiegend aus nicht infizierten Zellen bestanden haben müssen. Anomales, niedermolekulares Material unterliegt in noch viel stärkerem Masse dem Konzentrationseffekt bei der Sedimentation als dies bei den hochmolekularen TMV-Partikeln der Fall ist. Selbst radiomarkierte Substanz kann durch Adhäsion innerhalb der Masse der sedimentierenden normalen Zellpartikeln verschwinden und erst nach Erreichen einer gewissen Konzentration nachweisbar werden. Es wäre also durchaus möglich, dass die anomalen Substanzen schon vor oder gleichzeitig mit dem Auftreten des TMV vorhanden sind.

Wir versuchten diese Schwierigkeit zu umgehen, indem wir die Einzellenmethode, die wir schon seit Jahren für cytochemische Untersuchungen des Systems Wirtszelle–Virus verwendet hatten, auf die Auswertung der Extraktionen selbst übertrugen. Es stellte sich dabei heraus, dass die Masse isolierbarer Virussubstanz während eines grossen zeitlichen Abschnitts der Infektion (5.–8. IP) pro Zelle relativ konstant bleibt, dass aber der RNS-Gehalt dieser Nucleoproteide vom Beginn des Auftretens der TMV-Partikel an einem dauernden Schwund unterworfen ist. Dies stellt die bisherigen Untersuchungen vor eine gänzlich neue Situation. Unsere U.V.-spektrographischen Messungen *in situ* hatten schon früher die allmähliche Verschiebung des Gleichgewichts Virus-RNS–Virusprotein mit der Infektionsdauer nach rechts demonstrieren können. Diese Resultate stimmen mit gleichsinnigen Messungen an entsprechenden Zellextrakten überein. Wir waren allerdings früher der Auffassung, dass das Gleichgewicht durch andauernde Synthese des RNS-freien Virusproteinanteils verschoben würde, dass also Virus-RNS und Virus-Protein in verschiedenen Prozessen gebildet würden. Diese Vorstellung kann nicht aufrecht erhalten werden, man muss vielmehr annehmen, dass der RNS-Anteil der Virusmoleküle progressiv mit ihrer Alterung abgestossen wird, um dann wahrscheinlich im Stoffwechselgeschehen der Zelle abgebaut zu werden. Die stabileren Proteinhüllen bleiben noch längere Zeit erhalten. Da nur wenige infektive Einheiten genügen, die Nachbarzellen zu infizieren, scheint die Masse der Virusnachkommenschaft stationär in der Zelle zu verbleiben. Es sind keine experimentellen Hinweise dafür vorhanden, dass die sehr hohen Anzahlen der vermehrungsfähigen Einheiten des TMV andere schon infizierte Gewebe passieren und so schliesslich in nicht infizierte Regionen vordringen. Es konnte im Gegenteil gezeigt werden [25], dass präinfizierte Gewebe eine Barriere für die Durchwanderung des TMV darstellen können. Ebenso lassen sich Superinfektionen mit dem gleichen oder einem verwandten Stamm nicht durchführen. Es ist durchaus denkbar, dass ein Teil der Viruspartikel während ihres Transportes im strömenden Plasma der Zelle in Kontakt mit Receptoren der Nachbarzellen ihren vermehrungsfähigen Anteil abstossen, der aber nicht mehr in das benachbarte Cytoplasma eindringen kann. Für eine solche Deutung sprechen unsere Befunde, dass nach jeder der häufigen Auflösungen von virusbedingten Kristallen und nach ihrer Neubildung in irgendeiner Form deutliche RNS-Abnahme messbar war. Es entsteht nun die Frage, ob der Proteinmantel gleichfalls in der Zelle in kleinste Einheiten zerfällt und schliesslich über das X-Protein abgebaut wird. In Zellen der 9. IP ist bedeutend weniger und fast ausschliesslich RNS-freie TMV-Substanz nachweisbar. In Zellen vergilbender Blätter, die langfristig infiziert waren, konnten nur noch Reste des früher reichlich vorhandenen TMV-Proteins gefunden werden. Isoliert man andererseits Kristalle der letzten Infektionsperioden und kontrolliert leicht zerdrückte Teile davon im Elektronenmikroskop, so werden fast ausschliesslich lange Stäbchen in dichter Packung sichtbar. Wir konnten auch eindeutig nachweisen, dass ein beträchtlicher Teil der Virusstäbchen während der allgemeinen Sedimentierungsprozesse zerfällt.

Wie auch die Verhältnisse in Wirklichkeit liegen mögen, so ist doch sicher, dass mit steigendem Infektionsalter die isolierbare Menge RNS-freien TMV-Proteins zunimmt. Im Elektronenbild stellt sich diese Komponente stets in Bruchstücken verschiedenster Grössenordnung der ursprünglichen Teilchen dar. Noch intakte Teilchen können ebenfalls während der Extraktion bei neutralem pH ihren RNS-Zentralfaden ausstossen. Dieses geschieht möglicherweise im Kontakt mit einer gewebeeigenen Substanz. Danach zerfällt auch die Proteinhülle in die beschriebenen Bruchstücke und bis zu kleinsten Partikeln, die keine Strukturelemente des TMV mehr erkennen lassen. Vielleicht ist hier ein experimenteller Ansatz gegeben, den Infektionsprozess selbst aufklären zu können.

SUMMARY

Individual hair cells and one-cell layers of tobacco leaves were grouped according to their stage of infection with TMV and separate extracts of virus material were prepared by differential centrifugation.

Analyses of the virus extracts were carried out by electron microscopy, microspectrography and chemical methods. Results were expressed in relation to the stage of the TMV infection.

The results showed that the total mass of extracted virus substance per cell remained constant from the earliest appearance of the TMV particles until late in the infection at which time the extractable mass of virus substance per cell decreased slowly. It was concluded that the whole virus progeny within each cell was synthesized in a short time early in the course of the infection.

In confirmation of previous work, it was shown that during the course of the infection, the extracts contained a progressively increasing number of virus particles of decreased length due to fragmentation, and there was a simultaneous loss in their absorption at 2650 Å, their chemical RNA content and their infectivity.

Fragmentation and loss of RNA also occurred *in vitro* when a crude extract from tobacco leaf cells was mixed with a solution of mostly intact virus particles. The degree of fragmentation under these conditions was related to the stage of infection of the cells from which the virus was obtained.

Virus crystals studied *in situ* at a late stage of infection had a lower absorption at 2650 Å than those in earlier infected cells. The same crystals isolated by micromanipulation gave similar results both in the intact condition and in solution. Squashes prepared from isolated virus crystals showed particles predominantly of normal size, whether taken from early- or late-infected cells. However, when the crystals were dissolved, the majority of their component particles were observed to be fragmented in those taken from late-infected cells, while most particles from the early stages of infection remained unbroken.

Thus, fragmentation of the virus rods must have occurred after solution of the crystals. But changes in the fragility of these particles, which paralleled the stage of infection, developed earlier, probably while the particles were in contact with cellular substances. Both *in situ* and after isolation a number of TMV particles progressively increasing with the infection age lose their RNA. This change of the RNA / protein ratio is correlated with the increasing fragility of the extracted particles and the subsequent degradation of their protein-shells.

Wir danken Herrn Prof. Dr. Caspersson für die Ermöglichung und die grosszügige Förderung dieser Arbeit. Einer von uns (H. Z.) ist der Deutschen Forschungsgemeinschaft für die Gewährung eines Aufenthaltstipendiums zu grossem Dank verpflichtet.

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ON THE POSSIBLE ROLE OF HIGH ENERGY PHOSPHATE IN THE CORTICAL CHANGE OF SEA URCHIN EGGS

I. EFFECT OF DINITROPHENOL AND SODIUM AZIDE

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Upon fertilization the cortical granules embedded in the cortex of the unfertilized sea urchin egg are rapidly broken down in a wave-like fashion, starting at the site of sperm entry [12]. This change seems to cause elevation of the fertilization membrane, which may consist of the pre-existing vitelline membrane plus substances originating from the cortical granules [5, 12, 13, 14]. Evidence was recently presented by Sugiyama [15, 16] that the breakdown of the granules does not propagate *per se*, but results secondarily from a preceding invisible change which is self-propagating in nature ("fertilization wave"). Thus, according to him, the cortical change consists of a chain of reactions: Attachment of spermatozoon → Propagation of "fertilization wave" → Breakdown of cortical granules → Elevation of fertilization membrane.

Sugiyama's experiments indicated also that some activating agents, such as butyric acid, urea, distilled water, etc., cause the visible cortical changes, i.e. granule breakdown and membrane elevation, by inciting the fertilization wave in the same way that the spermatozoon does at the time of fertilization; while others, wasp-venom, sodium choleinate, Monogen, Lipon etc., provoke the visible changes without the intervention of the fertilization wave [15, 16]. Though it is still obscure, there may well be a certain relationship between the above chain of reactions and the sperm block (block to polyspermy) [17].

The object of the present series is to obtain some information concerning the mechanism underlying the cortical change at the biochemical level. Special attention was given to the possible participation of high energy phosphate in the chain reaction making up the cortical change. In this paper the effect of the uncouplers of oxidative phosphorylation, such as 2,4-dinitrophenol (DNP) and sodium azide (NaN_3), on the cortical change was investigated.

MATERIAL AND METHODS

Experiments were performed at the Sugashima Marine Biological Station from 1951 until 1954. The gametes of the sea urchins, *Heliocidaris crassispina, Temnopleurus toreumaticus* and *Pseudocentrotus depressus* served as the material. The spawning season of the two former species extends from July to August, while the third spawns from October to November.

The effect of pretreatment with the uncouplers on the cortical change was tested in the following manner. Unfertilized eggs were exposed to an uncoupler solution in sea water. After varying lengths of exposure, aliquots of the eggs were transferred to normal sea water and either immediately inseminated or subjected to a parthenogenetic treatment. The effect was examined with respect to membrane elevation, the sperm block and breakdown of the cortical granules. The influence on the sperm block, at fertilization and parthenogenesis, was judged from the incidence of polyspermy and the fertilizability after the parthenogenetic treatment, respectively. A control series was run in parallel by placing eggs from the same batch in normal sea water before insemination or parthenogenetic treatment. For insemination, 10^{-4} - 8×10^{-4} sperm suspension was used. The reproducibility of the results was tested by repeating the same experiment with eggs from different females. The percentage figures given below are based on counts done on 100–200 eggs.

RESULTS

Effect of pretreatment with DNP on the cortical change at fertilization (experiments with Heliocidaris and Temnopleurus eggs).—Pretreatment was carried out with 10^{-4}–5×10^{-4} *M* DNP solution in sea water (pH 8.0–8.3) at room temperature 25–32° C. Similar results were obtained with both species. Some of the typical results are shown in Tables I and II, from which it will be seen that DNP-pretreatment longer than a certain limited period (which ranged from 15 to 100 minutes) inhibits membrane elevation as well as the sperm block. Polyspermic eggs formed multiple sperm asters and either failed to cleave or cleaved irregularly. It is interesting to note that the inhibition of membrane elevation and of the sperm block took place almost in parallel. This may suggest some intimate relationship between the reaction underlying the two phenomena.

Observation of the cortical granules in *Temnopleurus* eggs with an oil immersion objective revealed that their breakdown upon fertilization was also prevented when the membrane elevation was completely inhibited by DNP-pretreatment. Thus the failure of the membrane elevation in DNP-treated eggs may be a result of the inhibition of granule breakdown.

If eggs which had been subjected to DNP for a sufficiently long time-period to bring about complete inhibition were replaced in normal sea water

TABLE I

Effect of DNP on the cortical change at fertilization (*Heliocidaris crassispina*). DNP-sea water: $10^{-4}M$, pH 8.2. Temperature: 27.0° C.

Methods of pretreatment	% of membrane elevation	% of polyspermy
No pretreatment	100	0
DNP-sea water (30 min.)	95	3
DNP-sea water (60 min.)	31	95
DNP-sea water (100 min.) . . .	18	98
DNP-sea water (120 min.) . . .	4	96
DNP-sea water (120 min.)		
→ Normal sea water (40 min.) . .	94	9
Normal sea water (160 min.). . .	100	2

TABLE II

Effect of DNP on the cortical change at fertilization (*Temnopleurus toreumaticus*). DNP-sea water: $2.5 \times 10^{-4}M$, pH 8.2. Temperature: 27.8° C.

Methods of pretreatment	% of membrane elevation	% of polyspermy
No pretreatment	100 (s.n. 3)	1
DNP-sea water (40 min.)	100 s.n. 10)	5
DNP-sea water (80 min.)	94 (s.n. 44)	61
DNP-sea water (100 min.) . . .	27 (s.n. 23)	97
DNP-sea water (120 min.) . . .	7 (s.n. 7)	97
DNP-sea water (120 min.)		
→ Normal sea water (100 min.). .	98 (s.n. 5)	0
DNP-sea water (220 min.) . . .	0	89
Normal sea water (285 min.). . .	98 (s.n. 11)	6

for varying periods (10–120 minutes) before insemination, reversal of the inhibition was observed, indicating that the DNP-inhibition of the cortical change is reversible, as shown in Tables I and II.

Summarizing, DNP-pretreatment reversibly inhibits the membrane elevation, the sperm block and breakdown of the cortical granules. It should be added, however, that the sensitivity to DNP differs considerably from batch to batch and that in a few cases (in 3 of 21 experiments with *Heliocidaris* and in 4 of 32 experiments with *Temnopleurus* the cortical change occurred almost normally, even after prolonged pretreatment.

Effect of DNP-pretreatment on the cortical change induced by butyric acid (experiments with Heliocidaris and Temnopleurus eggs).—If normal eggs of both species were exposed to butyric acid-sea water (6 and 4 per cent of *N*/10 butyric acid solution in sea water for *Heliocidaris* and *Temnopleurus* respectively) for 15–30 seconds and then rinsed in normal sea water, the granules were broken down, and membrane elevation occurred. The sperm block, which is essentially the same as that found upon fertilization, may also be produced by the butyric acid treatment, since eggs activated by butyric acid cannot be fertilized even if the membrane is mechanically removed [11].

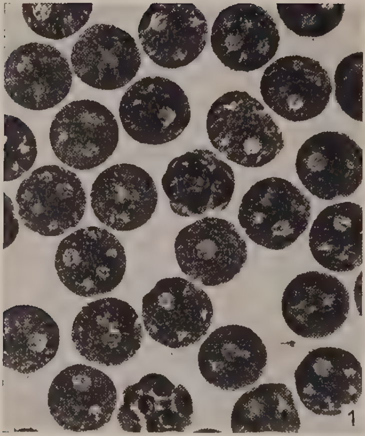
TABLE III

Effect of DNP on the cortical change induced by butyric acid (*Heliocidaris crassispina*). DNP-sea water: $10^{-4}M$, pH 8.1.

Methods of pretreatment	% of membrane elevation
No pretreatment	99
DNP-sea water (20 min.)	99
DNP-sea water (50 min.)	13
DNP-sea water (100 min.) . . .	0
DNP-sea water (100 min.) → Normal sea water (30 min.) .	93
Normal sea water (130 min.). . .	99

To test the effect of DNP on the cortical change induced by butyric acid, the eggs pretreated in the same way as in the above experiment were subjected to treatment with butyric acid. Table III shows a typical result. DNP-pretreatment was shown to block the membrane elevation as well as the granule breakdown induced by butyric acid treatment. It was also found that if the DNP-pretreated eggs which had failed to form fertilization membranes upon butyric acid treatment were subsequently inseminated, they later underwent cleavage, which was in many cases irregular. Cleavage did not take place, unless spermatozoa were added. Moreover, in certain cases even membrane elevation was found to occur upon such insemination. It is clear that spermatozoa can penetrate into these eggs. Therefore it is apparent that the sperm block, which would ordinarily take place upon butyric acid treatment, was inhibited by DNP-pretreatment.

In the majority of cases, the duration of DNP-pretreatment necessary for inhibition of the cortical change caused by butyric acid was the same as in the case of fertilization, so far as the eggs of the same batch were concerned. In some cases, however, a period of pretreatment shorter than that required in fertilization was sufficient to cause the inhibition of the cortical change upon treatment with butyric acid.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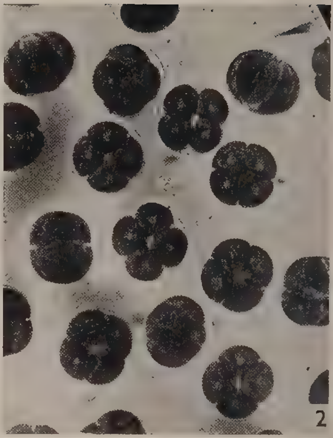
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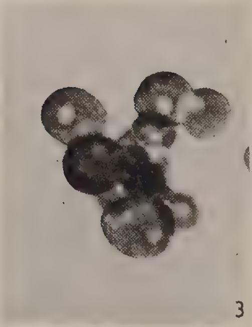
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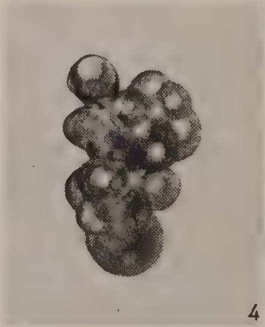
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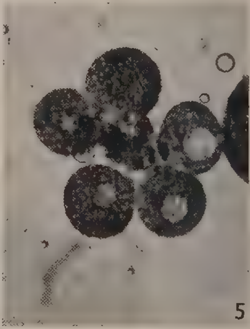
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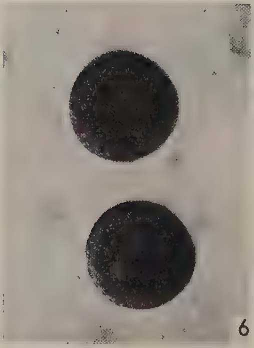
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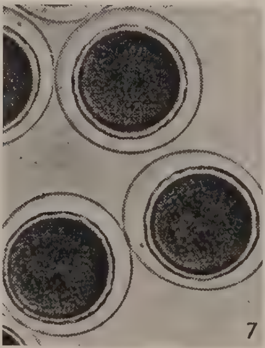
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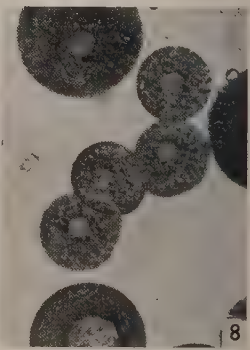
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Effect of DNP-pretreatment on the cortical change induced by urea (experiments with Heliocidaris eggs).—Treatment with molar urea solution for 3–6 seconds at room temperature (26–30° C) was effective in inducing the cortical change in *Heliocidaris* eggs. Experiments were carried out in the same manner as in the preceding experimental series. It was found that the membrane elevation, granule breakdown and the sperm block induced by urea are similarly inhibited in a reversible fashion by DNP-pretreatment. The length of the pretreatment needed for such inhibition, however, was shorter in this case than in fertilization and butyric acid activation. For a similar reason as in the case of butyric acid activation, it was concluded that DNP inhibits the sperm block on activation by urea. The DNP-pretreated eggs which had failed to form the membrane upon urea treatment could be fertilized. These elevated membranes when they were inseminated (5–30 minutes after urea treatment). In the majority of cases, the fertilization was monospermic, so that the eggs divided regularly.

It can be deduced from this observation that in the DNP-pretreated eggs, urea treatment does not cause any non-repeatable change in the chain of reactions leading to membrane elevation.

Effect of DNP-pretreatment on the cortical change induced by Monogen and sodium choleinate (experiments with Heliocidaris and Temnopleurus eggs).—0.03–0.2 per cent Monogen-sea water and 0.1–0.3 per cent sodium choleinate are effective inducers of the cortical change in both *Heliocidaris* and *Temnopleurus* eggs. DNP-pretreatment was done as usual and then the eggs were exposed to Monogen or sodium choleinate. Even after a DNP-pretreatment sufficiently prolonged to block completely the cortical change caused by spermatozoa, the eggs formed membranes upon exposure to these reagents. Moreover, the membrane elevation occurred somewhat faster in the DNP-pretreated eggs than in the control eggs, when both were exposed to the same concentration of these reagents. Thus the cortical change caused by Monogen or sodium choleinate is not inhibited, but rather stimulated by DNP-pretreatment.

Fig. 1. Eggs inseminated following DNP-pretreatment: no membrane formation, polyspermy.

Fig. 2. Normal membrane formation and division after the reversal of DNP-inhibition.

Figs. 3 and 4. Eggs inseminated following DNP-pretreatment: no membrane formation, polyspermy.

Fig. 5. An egg inseminated following NaN_3-pretreatment: no membrane formation, polyspermy.

Fig. 6. Eggs inseminated following DNP-pretreatment: partly elevated membrane (photographed 30 minutes after insemination).

Fig. 7. Control eggs with normal membrane and hyaline layer (photographed 30 minutes after insemination).

Fig. 8. An egg inseminated following DNP-pretreatment: no membrane, monospermy (central).

The hitherto described results may be summarized as follows: DNP-pretreatment inhibits the cortical change induced by spermatozoa, butyric acid and urea, while it does not inhibit the cortical change caused by Monogen and sodium choleinate. It is worth noting that the activating agents of the former group, the action of which is sensitive to DNP-pretreatment, correspond to the reagents causing, according to Sugiyama's hypothesis [16], the visible changes in the cortex through self-propagating fertilization wave. The latter agents, which are insensitive to DNP, are on the other hand those assumed to act on the cortical granules more directly without the intervention of the self-propagating change. This fact may provide the basis for determining the direct point of action of DNP in the chain of reactions constituting the cortical change. One of the most plausible possibilities is that DNP-pretreatment blocks the fertilization wave directly. Detailed discussion on this point will be presented later.

Influence of temperature on the DNP-inhibition of the cortical change in Pseudocentrotus eggs.—The inhibitory effect of DNP on the cortical change was first found in *Heliocidaris* and *Temnopleurus* eggs during the summer spawning season. The same experiments were repeated in autumn with *Pseudocentrotus depressus* eggs. In the earlier experiments, however, no effect of DNP was found even after prolonged pretreatment. Further investigation revealed, however, that if *Pseudocentrotus* eggs are exposed to DNP-sea water (2×10^{-4}–$5 \times 10^{-4} M$, pH 8.0–8.2) at 27–28° C, an inhibition of the cortical change upon fertilization is produced just as in *Heliocidaris* and *Temnopleurus* eggs. If the treatment is done at room temperature (14–20° C), no inhibitory effect appears. In the latter case, the eggs exposed to DNP-sea water are able to elevate the normal membrane as long as the control eggs kept in normal sea water maintain the capacity of membrane elevation. One typical experiment is given in Fig. 9.

Moreover, when eggs which had lost the capacity of forming the membrane as the result of previous DNP-treatment at a high temperature (e.g., 27° C) were put into DNP-sea water of room temperature (e.g., 18° C), reversal of the inhibition took place. These facts may imply that DNP only interferes with the cortical change at higher temperatures; in other words DNP-inhibition of the cortical change is cancelled at lower temperatures in this species. However, according to preliminary experiments, such a clear-cut temperature effect does not seem to exist in *Heliocidaris* and *Temnopleurus*.

Effect of NaN_3 on the cortical change upon fertilization (experiments with Heliocidaris, Temnopleurus and Pseudocentrotus eggs).—NaN_3 is known to inhibit oxidative phosphorylation [9] as does DNP [4, 8], while it blocks cytochrome oxidase [6] and adenosine triphosphatase [10] as well. If the inhibition of the cortical change produced by DNP be due to its uncoupling action on oxidative phosphorylation, NaN_3 might be expected to have a similar effect on the cortical change. To clarify this point, experiments similar to those with DNP were carried out using NaN_3 in the above three sea urchin species. For the pretreatment, $10^{-2}M$ NaN_3 solution in normal or artificial sea water was used. The pH of the solution was adjusted to 7.0 by adding a small amount of HCl, since it is known that NaN_3 is less effective on the alkaline side [2, 6]. Results indicated that just the same mode of inhibition of the cortical change is brought about by NaN_3 as by DNP. The membrane elevation, granule breakdown and the sperm block were inhibited reversibly. It was further found that NaN_3-inhibition of the cortical change is also cancelled at lower temperatures in the *Pseudocentrotus* eggs (see Fig. 10). Thus it seems very probable that DNP and NaN_3 block the cortical change through a common mechanism, i.e., uncoupling of oxidative phosphorylation.

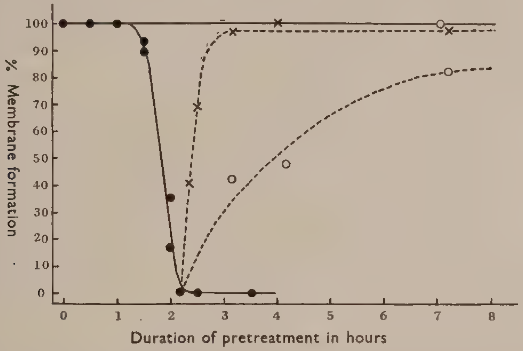

Fig. 9. Cancellation of DNP-inhibition of the cortical change by lower temperature in *Pseudocentrotus depressus* eggs. Changes in percentage membrane formation (upon fertilization) after
immersion in 5 × $10^{-4}M$ DNP-sea water at 27–28° C, —●———●—
immersion in 5 × $10^{-4}M$ DNP-sea water at 18° C, —○———○—
immersion in normal sea water at 27–28° C, —✕———✕—
transfer to normal sea water at 27–28° C following the DNP-treatment at 27–28° C, – ✕ – – – – ✕ –
transfer to DNP-sea water at 18° C following the DNP-treatment at 27–28° C, – ○ – – – – ○ –

Effect of addition of ATP on DNP-inhibition of the cortical change (experiments with Heliocidaris and Temnopleurus eggs).—If DNP exerts an inhibitory effect on the cortical change by blocking the oxidative generation of high energy phosphates including ATP, cancellation of the DNP-inhibition might be effected by the addition of ATP. Na_4-ATP (from Sigma Chemical Co.) was added to DNP-sea water so as to make its concentration approximately 2×10^{-4}–$10^{-3}M$. The pH of both the ATP-DNP-sea water and the DNP-sea water with which the eggs were treated prior to fertilization, was varied between 5.0 and 8.4. Within this pH range no appreciable effect of added ATP was found. ATP also failed to exert an accelerating effect on the reversal process of DNP-inhibition, when eggs previously subjected to DNP were placed in ATP-sea water instead of normal sea water.

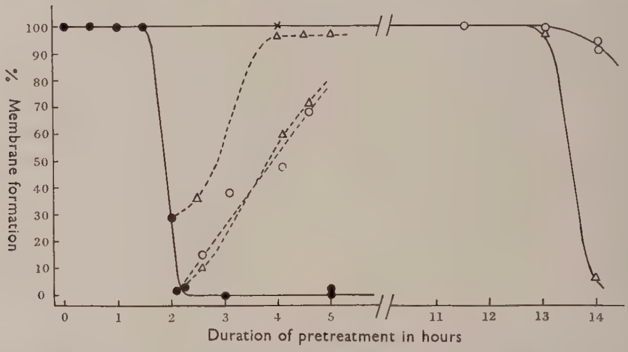

Fig. 10. Effect of NaN$_3$ on the cortical change upon fertilization in *Pseudocentrotus depressus* eggs. Changes in the percentage membrane formation after
immersion in $10^{-2}M$ NaN$_3$ at 27–28° C, —●———●—
immersion in normal sea water at 27–28° C, —✕———✕—
immersion in $10^{-2}M$ NaN$_3$-sea water at 18° C, —○———○—
immersion in normal sea water at 18° C, —△———△—
transfer to normal sea water at 18° C following the NaN$_3$-treatment at 27–28° C,
– △ – – – – △ –
transfer to NaN$_3$-sea water at 18° C following the NaN$_3$-treatment at 27–28° C,
– ○ – – – – ○ –

DISCUSSION

As described above, pretreatment with uncouplers, DNP and NaN$_3$, reversibly inhibits elevation of the fertilization membrane, breakdown of the cortical granules and the sperm block at the time of fertilization. Essentially the same inhibition was also observed with respect to the cortical change induced parthenogenetically by butyric acid or urea, whereas the cortical change caused by Monogen or sodium choleinate is not inhibited by these reagents.

In the light of Sugiyama's hypothesis [16], these results may indicate that the cortical change is inhibited by uncouplers, provided that it is induced through the fertilization wave. The cortical change caused without intervention of the fertilization wave, on the contrary, is not inhibited by the uncouplers. It follows that they block directly some step in the chain of reactions constituting the cortical change, which precedes the breakdown of the cortical granules, and without the intervention of which Monogen and sodium choleinate can induce the visible changes.

It was often observed that eggs previously treated with the uncouplers elevated the fertilization membrane within the small area around the site of sperm entry. In certain cases, where polyspermic fertilization took place such partial membrane elevation was found almost simultaneously in several places on the egg surface, each of which corresponded to the entrance of one spermatozoon. It may be inferred from these observations that the fertilization wave, which normally spreads over the entire egg surface starting from the point of sperm entry, is in such cases partially inhibited and propagates only within the restricted area around the entry point.

Furthermore, it may be assumed that, under sufficient influence of the uncoupler, butyric acid or urea does not cause any non-repeatable step in the chain of reactions leading to membrane elevation: DNP-pretreated eggs which have failed to elevate the membrane upon butyric acid or urea treatment are able to form the membrane when they are subsequently inseminated. This appears also to indicate that the uncouplers act on a very early step in the chain of reactions.

These observations strongly suggest that the direct point of attack of the uncouplers is the fertilization wave. However, at the same time we must consider the possibility that the uncouplers primarily block some other reaction, which also precedes the granule breakdown and all the non-repeatable changes. Without the intervention of this reaction Monogen and sodium choleinate may cause visible changes. Such a reaction might exist before or after the fertilization wave in the chain of reactions, although this has not yet been established. For example, it is possible that the uncouplers affect a reaction which acts as the trigger of the fertilization wave in the case of fertilization as well as in parthenogenesis by means of butyric acid or urea.

The participation of high energy phosphate in a biological process can be concluded from the observation that the process in question is inhibited by an uncoupler, provided the uncoupler acts in a specific way on oxidative phosphorylation. Strictly speaking, however, careful examination from various angles is necessary before any conclusion can be drawn, since there is usually no complete guarantee as to the specificity of an inhibitor.

As to the present case, the fact that the same mode of inhibition is produced by two kinds of uncouplers, DNP and NaN_3, lends support to the possibility that high energy phosphate participates in the cortical change. It is especially to be noted that the peculiar temperature effect in *Pseudocentrotus* eggs, i.e., cancellation of the inhibition of the cortical change at lower temperature, is common to both uncouplers. Other types of metabolic inhibitors so far tested are, on the other hand, not inhibitory to the cortical change (e.g., KCN and NaF) or else inhibitory both at high and low temperatures (e.g. iodacetamide). Thus it seems highly probable that DNP and NaN_3 inhibit the cortical change through the action common to both, i.e., the uncoupling of oxidative phosphorylation.

However, the observation that addition of ATP does not cancel the inhibition caused by DNP, may militate against the above assumption. According to Kriszat [7], DNP-inhibition of cleavage in *Psammechinus* eggs is to some extent counteracted by addition of ATP. Barnett [3] also showed with *Arbacia* eggs that the inhibition of division produced by cyanide and low oxygen tension is completely reversed when adding ATP, and that the inhibition caused by DNP can be partially relieved, although the NaN_3-inhibition of division is not at all affected by ATP. In view of these and other results, removal of the DNP-inhibition of the cortical change by addition of ATP might be expected to occur. The absence of this effect, however, might be explained in terms of the failure of the added ATP to penetrate into the cell or to reach the intracellular loci, where it is needed for the process in question. In spite of numerous studies using ATP in this field, it does not seem to the author to be perfectly established that ATP added to sea water can penetrate into unfertilized sea urchin eggs. It might also be conceivable that for some other reason ATP supplied externally cannot be used in the process as effectively as ATP generated within the cell. In addition, it was recently experienced that the DNP- and KCN-inhibition of cleavage in *Hemicentrotus pulcherrimus* was not affected by the addition of ATP preparation used in the present experiment. Therefore, the failure of added ATP to cancel DNP-inhibition of the cortical change is not necessarily an argument against the possibility that DNP inhibits the cortical change through its uncoupling action.

In favour of this possibility there is, in addition the fact that DNP-treatment causes considerable reduction, amounting to 30 per cent of the easily hydrolizable phosphate content of unfertilized eggs (preliminary data with *Pseudocentrotus* eggs).

In view of these considerations, it may be permissible to conclude tentatively that high energy phosphate participates in an early step of the cortical change—presumably in the fertilization wave. However, it is on the basis of the present experiments difficult to determine, whether phosphate bond energy is utilized directly in a reaction involved in the cortical change or only participates indirectly. The considerable lag in inhibition by DNP and NaN_3 might rather suggest the latter alternative, although it may also not be incompatible with the former, in view of the low metabolic activity of the unfertilized egg.

Recently Allen [1] made a similar suggestion, based on a different experimental basis. If *Psammechinus* eggs elongated in a glass capillary of small diameter are fertilized, visible changes in the cortex such as the breakdown of the cortical granules and a dark-field color change usually fail to cover the entire surface. Pretreatment of the eggs with ATP increases the area covered by these changes. This observation [1] is of special interest when taken in conjunction with the results reported in the present series. However, the following objections may be possible: in the first place, ATP-pretreatment might not really facilitate the cortical change but, presumably by increasing the protoplasmic rigidity, only prevent the impairment brought about by stretching and straightening of the egg surface and its contact with glass, as was in fact observed by Allen. In the second place, it should also be considered that facilitation of the visible progressive changes does not necessarily mean that of the selfpropagating invisible change. It is therefore not correct to conclude from his observation on the visible change, that ATP facilitates the passage of the impulse, i.e. the propagation of the fertilization wave. An alternative explanation may also be possible: ATP facilitates the granule breakdown directly so that the latter is induced even by a weak impulse or fertilization wave that would otherwise be insufficient. Consequently, the granules are broken even in the parts further away from the point of sperm entry, resulting in increase of the area covered by the visible changes.

In the present paper it is reported that in *Pseudocentrotus* eggs, the DNP- and NaN_3-inhibition of the cortical change are cancelled by lower temperature. This peculiar temperature effect might suggest that the change of temperature is accompanied by some qualitative rather than quantitative change in the metabolic pattern of the cell. The interesting mechanism underlying this cancellation of the inhibition remains to be investigated.

SUMMARY

1. Treatment of unfertilized eggs of the sea urchins, *Heliocidaris crassispina* and *Temnopleurus toreumaticus* with DNP and NaN_3 prior to insemination

was found to inhibit breakdown of the cortical granules, elevation of the fertilization membrane and the sperm block at the time of fertilization. This inhibition of the cortical change by the uncouplers is reversible.

2. Essentially the same inhibitory effect was observed on the cortical change parthenogenetically induced by butyric acid or urea.

3. The uncouplers, however, do not block the cortical change induced by Monogen or Na-choleinate.

4. In *Pseudocentrotus depressus,* when the pretreatment is carried out at lower temperatures (14–20° C), neither DNP nor NaN_3 is inhibitory; but at higher temperatures (27–29° C), their inhibitory effect on the cortical change is always apparent. Moreover, when eggs are subjected to these uncouplers at a higher temperature for a sufficient time-period to produce almost complete inhibition, and are thereafter placed into sea water of lower temperature containing the uncouplers, a reversal of the inhibition takes place.

5. Addition of ATP to DNP-sea water does not remove the inhibition produced by DNP.

6. It is suggested that the uncouplers primarily act on some of the early steps of the chain of reactions making up the cortical change, presumably on the fertilization wave, in which high energy phosphate may participate.

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II. EFFECT OF URANYL NITRATE

In a series of studies Rothstein and his coworkers [4, 7, 8, 9, 10] have shown that uranyl nitrate (UN) at 10^{-3}–$10^{-5}\,M$ inhibits the active transport of glucose in yeast cells, presumably by forming a complex with polymerized phosphate located on the cell surface. On the other hand, it was reported by Villee *et al.* [13] that UN inhibits also the incorporation of P^{32} into developing sea urchin eggs, which, according to Lindberg [5], occurs through oxidative phosphorylation on the cell surface.

Experiments reported in the preceding paper [6] suggested the possibility that high energy phosphate participates in one of the early steps in the chain of reactions which constitute the cortical change of sea urchin eggs. It would therefore seem to be of special interest to inquire whether UN blocks the cortical change. To elucidate this point, two lines of experiments were undertaken. In the first place, the effect of a treatment of eggs with UN prior to insemination or parthenogenetic treatment was tested in the same way as in the previous experiments with DNP and NaN_3. In the second place, the direct effect of the presence of UN in the medium at the time of activation was investigated. A study of the effect of $Ba^{\cdot\cdot}$ on the cortical change will also be reported here. The results obtained seem to provide further support for the above hypothesis.

MATERIAL AND METHODS

The gametes of the sea urchins *Temnopleurus toreumaticus* and *Heliocidaris crassispina* were used. Essentially the same results were obtained with both species. Experiments were carried out at the Sugashima Marine Biological Station during the summer 1954.

UN was stored in a $10^{-2}M$ solution in distilled water, which was diluted with sea water to obtain appropriate concentrations for the experiments (10^{-4}–5×10^{-4} M). The pH of the solution was adjusted by adding a small amount of HCl and NaOH. Insemination was performed with 10^{-3}–10^{-4} sperm suspension. The butyric acid solution used for parthenogenesis was prepared by mixing 4 volumes of $N/10$ butyric acid with 96 volumes of sea water for *Temnopleurus*, and 6 volumes of $N/10$ butyric acid with 94 volumes of sea water for *Heliocidaris*. The optimum duration of exposure of eggs to butyric acid-sea water, which ranged from 15 to 30 seconds, was determined separately for each batch. The concentrations of the Monogen- and sodium choleinate-sea water used for activation were 0.03–0.1 per cent and 0.1–0.3 per cent, respectively. All the experimental procedures were conducted at room temperature (25–30.5° C).

RESULTS

Effect of pretreatment with UN.—Eggs were exposed for varying periods to 10^{-4}–$5 \times 10^{-4} M$ UN-sea water (pH 5.5–7.0) prior to fertilization. Insemination was carried out in normal sea water immediately following the pretreatment. In these eggs the penetration of spermatozoa occurred successfully, but the cortical change was blocked. Table I shows one of typical results. It will be seen that the inhibition became more and more intensive as the duration of pretreatment was prolonged. Fifteen minutes' pretreatment caused only a delay in membrane elevation, whereas after 30 or 60 minutes pretreatment the percentage of membrane elevation was markedly decreased and the delay was much more pronounced. In the latter cases the membrane was always subnormal, i.e. low, less refractive and sometimes only locally elevated. A pretreatment longer than 95 minutes completely abolished the membrane elevation. The percentage of polyspermic fertilization increased in parallel. If eggs pretreated with UN for 30 or 60 minutes were put into normal sea water for more than 20 minutes, and then inseminated, the cortical change

TABLE I[1]

Effect of UN-pretreatment on the cortical change at fertilization (*Temnopleurus toreumaticus*). UN.-s.w.: $4 \times 10^{-4} M$, pH 6.4. Temperature 27.7° C.

Method of pretreatment	Length of time taken from insemination until the the beginning of membrane elevation	% membrane elevation	% polyspermy
No pretreatment	18–22 sec.	100 (s.n. 0)	2
UN-s.w. (15 min.)	70–90 sec.	97 (s.n. 7)	14
UN-s.w. (30 min.)	4 min.–6 min. 45 sec.	23 (s.n. 23)	96
UN-s.w. (60 min.)	6 min. 20 sec.– 8 min. 10 sec.	12 (s.n. 12)	97
UN-s.w. (95 min.)	∞	0	100
UN-s.w. (125 min.)	∞	0	95
UN-s.w. (30 min.) → n.s.w. (22 min.)	20–30 sec.	96 (s.n. 12)	11
UN-s.w. (60 min.) → n.s.w. (25 min.)	20–30 sec.	96 (s.n. 12)	10

[1] The following abbreviations have been used in Tables I–X.
UN-s.w.: uranyl nitrate-sea water
n.s.w.: normal sea water
s.n.: subnormal membrane
B.a.-s.w.: butyric acid-sea water
UN-b.a.-s.w.: uranyl nitrate-butyric acid-sea water

took place almost normally. This indicates that the inhibition of the cortical change produced by UN-pretreatment is reversible unless the treatment is too extended.

The cortical change parthenogenetically induced by butyric acid was also blocked by UN-pretreatment, but that caused by Monogen or sodium choleinate was not inhibited at all. Thus the inhibitory effect of UN-pretreatment is identical with those of DNP and NaN_3 in most respects examined. The only difference noticed was that the delay in membrane elevation was more pronounced in the case of UN-inhibition than in the case of DNP and NaN_3. In the former, subnormal membranes occasionally appeared even 5–10 minutes after insemination; while in the latter, the membranes were usually not formed at all, unless they appeared within the first 3 minutes subsequent to insemination.

Assuming that spermatozoa and butyric acid indirectly cause the visible cortical changes through the intervention of the fertilization wave, while Monogen and sodium choleinate cause these changes without its participation, it may be said that UN-pretreatment blocks some early step of the chain of reactions constituting the cortical change, just as do DNP and NaN_3.

A pretreatment with UN-sea water at pH 8.2 was not inhibitory to the cortical change.

The cortical change on fertilization in the presence of UN.—If eggs and spermatozoa were put into $5 \times 10^{-4} M$ UN-sea water (pH 7.8–8.0) simultaneously, fertilization took place but the cortical change was impaired to a certain extent. Table II shows one of the results, where almost complete inhibition of the cortical change was observed. In some batches the inhibitory effect of UN was less marked. If the pH of UN-sea water was slightly lowered, the inhibition became more complete. However, this does not necessarily mean that the inhibitory action of UN *per se* is intensified by lowering the pH, since it was found in the control that a pH slightly lower than that of normal sea water alone impairs the cortical change to some extent. At a

TABLE II

Effect of the presence of UN on the cortical change at fertilization (*Heliocidaris crassispina*). UN-s.w.: $5 \times 10^{-4} M$, pH 8.0.

Method of treatment	% membrane elevation	% polyspermy
Insemination in n.s.w.	100 (s.n. 0)	0
,, in UN-s.w. . . .	0	90

pH lower than 7.5, UN immobilized the spermatozoa so that fertilization failed to occur.

Effect of the presence of UN on the cortical change induced by butyric acid. —As mentioned above, the presence of UN in the medium blocks the cortical change at the time of fertilization. However, it must be asked whether UN acts on the cortex of the egg directly. Since UN has inhibitory effects on the respiration [1] as well as on the movement of sea urchin spermatozoa, the inhibition of the cortical change upon fertilization by UN might be due to its influence on the spermatozoa. The question may be answered, at least to a certain extent, by investigating the effect of UN on the cortical change induced by parthenogenetic agents. Experimentation revealed that the presence of UN blocks the cortical change induced by butyric acid also. The inhibition is more pronounced here than in the case of fertilization. Therefore it may be concluded that UN blocks directly some reaction occurring in the egg which underlies the cortical change.

The procedure of artificial activation by means of butyric acid consists of two steps: (1) exposing the eggs to butyric acid-sea water, (2) washing the eggs with normal sea water. The visible changes such as cortical granules breakdown and membrane elevation do not take place during the first step and a second step is therefore indispensable. This may imply that in the presence of butyric acid the chain reactions making up the cortical change are initiated, but do not proceed beyond a certain point, and that the butyric acid must be removed in order to secure further progress. Therefore, the chain reactions may be divided into two phases—an early phase which takes place in the presence of butyric acid (first phase) and a later one which is blocked in butyric acid-sea water, but proceeds after washing the eggs with normal sea water (second phase). However, it is also possible that the reactions of the first phase which start during the first step treatment occur even after transfer to normal sea water.

In order to determine the phase to which the UN-sensitive reaction belongs, one or both of the two steps of the treatment were carried out in the presence of UN, viz.:

(a) UN-butyric acid-sea water → Normal sea water;
(b) Butyric acid-sea water → UN-sea water;
(c) UN-butyric acid-sea water → UN-sea water.

In Series (a), where the first step treatment alone was carried out in the presence of UN, weak inhibition was usually revealed by the delay and subnormality of membrane elevation. On the other hand, if the second step treatment was done in the presence of UN (Series (b)), much more pronounced inhibition of the membrane elevation was observed. The most complete inhibition was found in Series (c), where both steps of the activation procedure were performed in the presence of UN. In Tables III and IV some of the representative results are given.

TABLE III

Effect of UN on the cortical change induced by butyric acid (*Heliocidaris crassispina*). Duration of the first step treatment: 30 seconds. UN concentration in UN-b.a.-s.w.: $10^{-3}M$. UN-s.w.: $4 \times 10^{-4}M$, pH 8.3. Temperature: 25.8° C.

Method of treatment	Length of time taken from the end of the first step treatment until the beginning of membrane elevation	% membrane elevation
B.a.–s.w. → n.s.w.	35–55 sec.	99 (s.n. 0)
UN-b.a.-s.w. → n.s.w.	60–100 sec.	99 (s.n. 23)
B.a.-s.w. → UN-s.w.	125–180 sec.	38 (s.n. 38)
UN-b.a.-s.w. → UN-s.w.	∞	0

TABLE IV

Effect of UN on the cortical change induced by butyric acid (*Temnopleurus toreumaticus*). Duration of the first step treatment: 20 seconds. UN concentration in UN-b.a.-s.w.: $10^{-3}M$. UN-s.w.: $5 \times 10^{-4}M$, pH 8.3. Temperature: 28.5° C.

Method of treatment	Length of time taken from the end of the first step treatment until the beginning of membrane elevation	% membrane elevation
B.a.-s.w. → n.s.w.	15–20 sec.	99 (s.n. 1)
UN-b.a.-s.w. → n.s.w.	25–35 sec.	100 (s.n. 2)
B.a.-s.w. → UN-s.w.	∞	0
UN-b.a.-s.w. → UN-s.w.	∞	0

From these results it appears that UN acts mainly on some reaction of the second phase, although some influence of UN on the first phase reactions is not excluded. However, the possibility must be considered that UN added to butyric acid-sea water is adsorbed on the egg surface and not perfectly removed after the washing, thereby influencing the second phase reaction to some extent. In this case the influence of UN on the first phase need not be considered. It is also conceivable that UN acts on some reaction which starts during the first step treatment, continues, and becomes more active after washing the eggs with normal sea water.

In Series (b) and (c), the breakdown of the cortical granules was also found to be more or less completely inhibited. Furthermore, if the eggs which had failed to form the membrane under the influence of UN were inseminated in normal sea water 5–10 minutes after butyric acid treatment, no membrane elevation took place. But multiple asters were later formed, indicating the occurrence of polyspermic fertilization. Thus it is evident that UN inhibits also the sperm block which would take place upon activation by butyric acid.

It is worth noting that in Series (b) and (c) subnormal membranes occasionally appeared after a pronounced delay; for instance, in *Temnopleurus* eggs, the membrane might appear 3–15 minutes subsequent to the end of the first step treatment in Series (b) and (c), whereas in the absence of UN the membrane elevation started 12–20 seconds after the end of the first step treatment (see Figs. 5 and 6). This may suggest that under the influence of UN the chain reactions which lead to membrane elevation proceed at a strongly declining rate and in many cases are stopped before giving rise to the visible changes.

In this connection the results of the following experiment will be of interest. Eggs were previously treated in the same way as in Series (b) or (c), but transferred to normal sea water after a short exposure to UN-sea water, as follows:

Butyric acid-sea water → UN-sea water → Normal sea water; or UN-butyric acid-sea water → UN-sea water → Normal sea water. The duration of exposure to UN-sea water was varied from 20 to 180 seconds. Two representative results are shown in Tables V and VI. In the case shown in Table V, if the eggs were treated in the same way as in Series (c) throughout, the membrane elevation failed completely to take place, while if the eggs were put into normal sea water within 120 seconds after their transfer from UN-butyric acid to UN-sea water, the membrane was elevated. However, in these cases the length of time taken from transfer of the eggs to normal sea water until the beginning of membrane elevation was longer, the membrane elevation was less normal and less frequent than in the case of eggs previously exposed to UN-butyric acid-sea water and transferred directly to normal sea water. Furthermore, as will be seen from Table V, the longer the eggs were exposed to UN-sea water before transfer to normal sea water, the more pronounced was the delay and subnormality of the membrane elevation. Eggs exposed to UN-sea water for more than 150 seconds failed to elevate the membrane even after transfer to normal sea water. A similar relation holds true in the case given in Table VI. In this case, however, 40 seconds' exposure to UN-sea water was sufficient to block the membrane elevation after transfer to normal sea water.

These results may best be explained by supposing that if UN, which acts like a brake on the progress of the chain reactions leading to membrane

TABLE V

Effect of UN on the cortical change induced by butyric acid (*Temnopleurus toreumaticus*). UN concentration in UN-b.a.-s.w. and UN-s.w.: $5 \times 10^{-4}M$. PH of UN-s.w.: 8.3. Temperature: 28.2° C.

Method of treatment	Length of time taken from the transfer of eggs to n.s.w. until the beginning of membrane elevation	% membrane elevation
B.a.-s.w. (15 sec.) → n.s.w. . . .	15–20 sec.	98 (s.n. 7)
UN-b.a.-s.w. (15 sec.) → n.s.w. . .	25–40 sec.	96 (s.n. 8)
UN-b.a.-s.w. (15 sec.) → UN-s.w. .	∞	0
UN-b.a.-s.w. (15 sec.) → UN-s.w. (30 sec.) → n.s.w.. . .	45–110 sec.	95 (s.n. 7)
UN-b.a.-s.w. (15 sec.) → UN-s.w. (120 sec.) → n.s.w. . .	70–140 sec.	92 (s.n. 86)
UN-b.a.-s.w. (15 sec.) → UN-s.w. (120 sec.) → n.s.w. . .	180–210 sec.	15 (s.n. 15)
UN-b.a.-s.w. (15 sec.) → UN-s.w. (150 sec.) → n.s.w. . .	∞	0

TABLE VI

Effect of UN on the cortical change induced by butyric acid (*Temnopleurus toreumaticus*). UN-s.w.: $5 \times 10^{-4}M$, pH 8.3. Temperature: 28.5° C.

Method of treatment	Length of time taken from the transfer of eggs to n.s.w. until the beginning of membrane elevation	% membrane elevation
B.a.-s.w. (20 sec.) → n.s.w. . . .	15–20 sec.	99 (s.n. 1)
B.a.-s.w. (20 sec.) → UN-s.w. . .	∞	0
B.a.-s.w. (20 sec.) → UN-s.w. (20 sec.) → n.s.w. . . .	50–60 sec.	87 (s.n. 76)
B.a.-s.w. (20 sec.) → UN-s.w. (30 sec.) → n.s.w. . . .	60–120 sec.	25 (s.n. 25)
B.a.-s.w. (20 sec.) → UN-s.w. (40 sec.) → n.s.w. . . .	∞	0
B.a.-s.w. (20 sec.) → UN-s.w. (60 sec.) → n.s.w. . . .	∞	0

TABLE VII

Effect of UN on the cortical change induced by butyric acid (*Temnopleurus toreumaticus*). UN-s.w.: $5 \times 10^{-4} M$, pH 8.3. Temperature: 27.0° C.

Method of treatment	Length of time taken from the end of the first step treatment until the beginning of membrane elevation	% membrane elevation
i) B.a.-s.w. (25 sec.) → n.s.w. . .	12–17 sec.	100 (s.n. 0)
ii) UN-b.a.-s.w. (25 sec.) → n.s.w.	30–40 sec.	100 (s.n. 0)
iii) UN-b.a.-s.w. (25 sec.) → UN-s.w.	∞	0
iv) Un-b.a.-s.w. (25 sec.) → n.s.w. (18 sec.) → UN-s.w.	40–70 sec.	98 (s.n. 75)

elevation, is removed before stopping their progress completely or slowing it down sufficiently, the rate of the reactions recovers to some extent and the membrane is elevated.

In view of the above considerations, it is tempting to assume that what is directly blocked by UN is not some reaction involved in the visible changes of the cortex but one which precedes them, although it may belong to the second-phase reactions. The results of the next experiment can also be interpreted as supporting this idea. Eggs were treated as follows:

UN-butyric acid-sea water → Normal sea water → UN-sea water. The results are shown in Table VII. In this batch, when the first step treatment alone was done in the presence of UN, the membrane began to elevate 30–40

Fig. 1. Eggs inseminated following UN-pretreatment: elevation of subnormal membranes 4 to 8 minutes after insemination (photographed 12 minutes after insemination).

Fig. 2. Eggs inseminated following UN-pretreatment: no membrane formation, polyspermy (photographed 1 hour after insemination).

Fig. 3. Eggs treated first with butyric acid-sea water, and then transferred to normal sea water: normal membrane elevation (photographed 7 minutes after transfer to normal sea water).

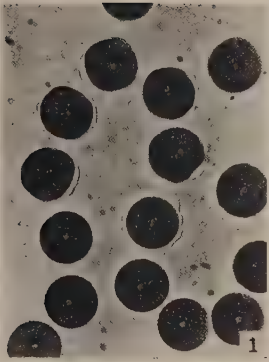
Fig. 4. Eggs treated first with UN-butyric acid sea water, and then transferred to UN-sea water: no membrane formation (photographed 5.5 minutes after transfer to UN-sea water).

Figs. 5 and 6. Eggs treated first with UN-butyric acid sea water, and then transferred to UN-sea water, showing much delayed membrane elevation. *Fig. 5*: Photograph taken 8 minutes after transfer to UN-sea water, showing no elevated membrane. *Fig. 6*: Photograph of the same microscopic field taken 15 minutes after transfer to UN-sea water, showing elevated membranes.

Fig. 7. Eggs treated first with UN-butyric acid sea water, and then with UN-sea water (for 30 seconds) and transferred to normal sea water (photographed 6 minutes after transfer from UN-butyric acid-sea water to UN-sea water).

Fig. 8. Eggs treated first with butyric acid sea water and then with $Ba^{\cdot\cdot}$-sea water: no membrane formation (photographed 6 minutes after transfer to $Ba^{\cdot\cdot}$-sea water).

Fig. 9. Eggs treated first with butyric acid-sea water and then with NaH_2PO_4-$Ba^{\cdot\cdot}$-sea water: Normal membrane formation (photographed 5 minutes after transfer to NaH_2PO_4-$Ba^{\cdot\cdot}$-sea water).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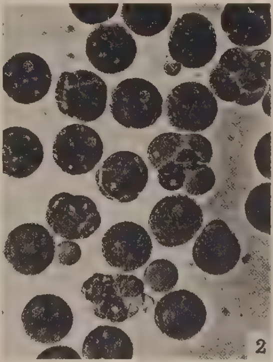
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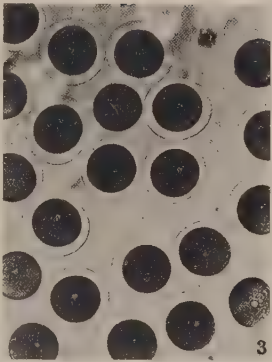
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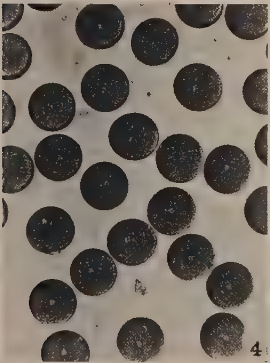
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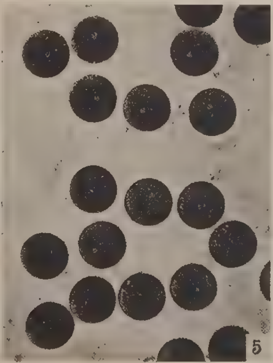
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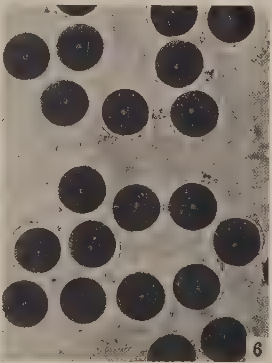
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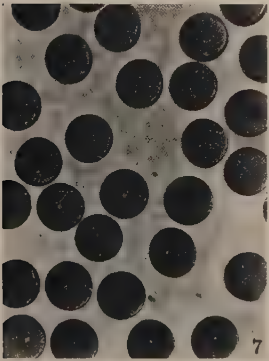
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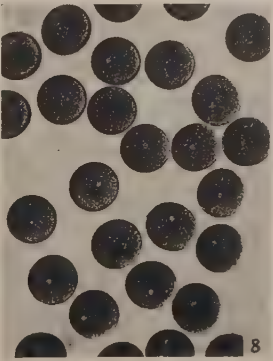
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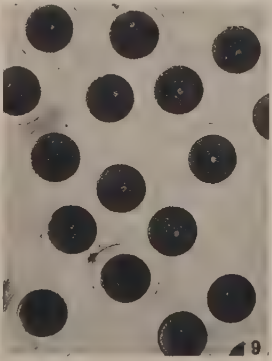
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TABLE VIII

Removal of the inhibitory action of UN on the cortical change accompanying butyric acid treatment by addition of ATP (*Heliocidaris crassispina*, the same batch as Table III). Duration of the first step treatment: 30 seconds. UN concentration in UN-b.a.-s.w.: $10^{-3}M$. UN-s.w.: $4 \times 10^{-4}M$, pH 8.3. ATP-UN-s.w.: prepared by adding 1 ml $8 \times 10^{-3}M$ ATP to 20 ml $4 \times 10^{-4}M$ UN-s.w. and removing the precipitate; pH 8.3. Temperature; 25.8° C.

Method of treatment	Length of time taken from the end of the first step treatment until the beginning of membrane elevation	% membrane elevation
B.a.-s.w. → n.s.w.	35–50 sec.	99 (s.n. 0)
UN-b.a.-s.w. → n.s.w.	60–110 sec.	99 (s.n. 23)
B.a.-s.w. → UN-s.w.	125–180 sec.	38 (s.n. 38)
UN-b.a.-s.w. → UN-s.w.	∞	0
B.a.-s.w. → ATP-UN-s.w.	35–50 sec.	100 (s.n. 0)
UN-b.a.-s.w. → ATP-UN-s.w. . .	60–110 sec.	99 (s.n. 17)

seconds after the transfer of the eggs to sea water (Series ii in Table VII). The reaction involved in the visible changes may be inferred to take place in the second half of this 30–40 second lag. In Series iv the eggs were transferred to UN-sea water in the middle of this lag, i.e. after 18 seconds' exposure to normal sea water. A prolongation of the time span between the transfer to normal sea water and the beginning of membrane elevation was observed in comparison with the data of Series ii. On the other hand, if the eggs were treated throughout the first and second steps in the presence of UN (Series iii), the membrane failed to appear. In view of the above-mentioned fact that the cortical change is blocked when eggs are exposed to UN-sea water only 12–20 seconds before the expected beginning of the membrane elevation, it appears that UN acts instantaneously. Therefore the difference in the results between Series ii and iv is to be accounted for in terms of the effect of UN on the later part of the reaction chain, which involves the reactions directly underlying the visible changes. The difference in the results between Series iii and iv, on the other hand, may be ascribed to the effect of UN on reactions which are not involved in the visible changes. Results reveal that the difference between Series ii and iv is small, if any, whereas the membrane elevation is much improved in Series iv in comparison with Series ii. It follows that UN attacks a reaction which takes place mainly during the first half of the period from the beginning of the second step treatment to the starting of the membrane elevation; i.e., some reaction which is not directly involved in the visible cortical changes.

When 1 ml $8 \times 10^{-3} M$ ATP-sea water was added to 20 ml $3 \times 10^{-4} M$ UN-sea water, a considerable amount of white precipitate was formed, which might be regarded as uranyl-ATP complex. The supernatant of this ATP-UN-sea water was adjusted to pH 8.2 and used for the second step treatment of butyric acid activation. It was found, as will be seen in Table VIII, that the supernatant thus prepared fails altogether to interfere with the cortical change caused by butyric acid. The same was found when excess Na_2HPO_4 instead of ATP was added to UN-sea water. These facts may suggest the possibility that a similar complex formation of uranyl ions with phosphate compounds is involved in the mechanism by which UN inhibits the cortical change.

Effect of the presence of UN on the cortical change caused by Monogen and sodium choleinate.—Table IX shows the result of the experiment concerning the effect of UN on the cortical change induced by Monogen. It will be seen that UN exerts no effect on either the percentage of membrane elevation or the duration of exposure of eggs to Monogen needed to initiate membrane elevation. The same is true of membrane elevation caused by sodium choleinate. These results may indicate, in agreement with the suggestion made previously, that the reactions which are directly involved in the visible change are not attacked by UN, but that some reaction which precedes the visible change is sensitive toward UN.

TABLE IX

Effect of UN on the cortical change induced by Monogen (*Heliocidaris crassispina*). Temperature: 30.5° C.

Method of treatment	Length of time taken from the beginning of treatment until the starting of membrane elevation	% membrane elevation
0.1% Monogen-s.w.	15–20 sec.	100
0.1% Monogen-$4 \times 10^{-4} M$ UN-s.w.	15–20 sec.	100
0.03% Monogen-s.w.	45–140 sec.	87
0.03% Monogen-s.w.-$4 \times 10^{-4} M$ UN-s.w.	45–145 sec.	88

Effect of $Ba^{\cdot\cdot}$ on the cortical change in fertilization and butyric acid activation.—The experiments hitherto described tend to indicate that high energy phosphate plays a role in the cortical change on the cell surface and that UN blocks the cortical change by forming a complex with it. If this is really the case, $Ba^{\cdot\cdot}$, which also forms a complex with ATP, inorganic phosphate, etc. would be expected to exert similar inhibitory effect on the cortical change.

Upon experimentation, however, a difficulty arose from the fact that sea water contains a large amount of SO_4'', which forms insoluble $BaSO_4$ when $Ba^{\cdot\cdot}$ is added. Therefore, the experiment was carried out in the following way. First isotonic $BaCl_2$ or Ba-acetate was added to sea water step by step, the precipitate being removed by centrifugation, until no more precipitate was formed. The supernatant solution thus prepared can scarcely contain both SO_4'' and $Ba^{\cdot\cdot}$. Then a certain volume of isotonic $BaCl_2$ or Ba-acetate was added further so as to make the concentration of $Ba^{\cdot\cdot}$ 10^{-3}–$5 \times 10^{-3}M$. The solution obtained may be regarded as 10^{-3}–$5 \times 10^{-3}M$ $Ba^{\cdot\cdot}$ (SO_4''-free) sea water. If insemination was carried out in this solution, fertilization occurred, but the cortical change was incompletely blocked. Delay and lowering of the percentage of membrane elevation was found, and the membranes elevated were subnormal. Similar, but somewhat more pronounced, inhibition of the cortical change was observed when the second step treatment of the butyric acid activation was done with this solution (see Table X). It is, however, obscure whether the observed effect is due to the presence of $Ba^{\cdot\cdot}$ or to the absence of SO_4''. To clarify this point, $Ba^{\cdot\cdot}$ was removed from this solution by adding excess Na_2HPO_4, and precipitating it as barium phosphate. Then the effect of the supernatant solution lacking in both $Ba^{\cdot\cdot}$ and SO_4'' was tested. It was found that the $Ba^{\cdot\cdot}$-SO_4-free sea water exerts no inhibitory effect on the cortical change. It is thus evident that the inhibitory effect is due to the presence of $Ba^{\cdot\cdot}$, but not to the absence of SO_4''.

TABLE X

Effect of $Ba^{\cdot\cdot}$ on the cortical change induced by butyric acid (*Temnopleurus toreumaticus*). Duration of the first step treatment: 20 seconds. $Ba^{\cdot\cdot}$-s.w. ($-SO_4''$): ca. $3.5 \times 10^{-3}M$ $BaCl_2$-SO_4''-free sea water (pH 8.3). P-$Ba^{\cdot\cdot}$-s.w.($-SO_4''$): prepared by adding 50 mg $NaH_2PO_4 12H_2O$ to 30 ml. $Ba^{\cdot\cdot}$-s.w.($-SO_4''$) and removing the precipitate; pH 8.3. Temperature: 28.2° C.

Methods of treatment	Length of time taken from the end of the first step treatment to the beginning of membrane elevation	% membrane elevation
B.a.-s.w. → n.s.w.	15–20 sec.	98 (s.n. 0)
B.a.-s.w. → $Ba^{\cdot\cdot}$-s.w. ($-SO_4''$)[3] .	∞	0
B.a.-s.w. → P–$Ba^{\cdot\cdot}$-s.w. ($-SO_4''$)[4]	15–20 sec.	100 (s.n. 1)

DISCUSSION

UN is known to inhibit the glucose metabolism of yeast cells [1, 2, 10]. The mechanism of this UN-inhibition of glucose metabolism in yeast was extensively investigated by Barron *et al.* [1] and Rothstein *et al.* [4, 7, 8, 9, 10]. The investigations of these two groups of workers agree in indicating that the UN-inhibition is due, not to direct interference with the fermentative metabolism within the cell, but to the formation of UN complex with the cell surface, and thereby interfering with the transport of glucose. For example, it was shown that if UN is added earlier than or simultaneously with glucose, the effect is instantaneous, while if it is added 10 minutes after the addition of glucose, a considerable delay occurs in the appearance of inhibition [10]. Moreover, UN has no effect on endogenous fermentation induced by DNP [10]. However, there exists a discrepancy of opinion between the two schools as to the chemical nature of the uranyl complex formed with the cell surface. Barron *et al.* suggested that the uranyl ions form complexes with proteins of the cell membrane, thereby reducing the permeability of the cells to sugars. On the other hand, Rothstein's group, criticizing this idea, postulated that the uranyl complexing loci of the cell surface are polymers of phosphate which are presumably necessary for phosphorylation reactions involved in the active transport of glucose into the cell. The hypothesis proposed by Rothstein's group is mainly based on two lines of experimental research—a kinetic study of UN-inhibition of glucose metabolism [4] and a detailed comparison between the chemical properties of the uranyl-cell surface complex and uranyl complexes with a large variety of known biochemical substances [10]. These studies established, first, that the kinetics of UN-inhibition are typical of those expected in an enzyme reaction, and not of a diffusion-limited process. And secondly it was revealed that the polyphosphates, among various substances, have chemical similarities with the loci of the cell surface in the order of stability of the uranyl complexes as well as in their behaviour toward pH.

These observations on the effect of UN on the cell surface of yeast should not be automatically extended to other cells, e.g. marine eggs. However, there are some reasons to believe that a similar situation prevails also in sea urchin eggs. For example, Vilee *et al.* [13] found that UN prevents approximately 50 per cent incorporation of P^{32} into developing sea urchin eggs. On the other hand, it was shown by Lindberg's work [5] that the incorporation of P^{32} into sea urchin eggs involves phosphorylation reactions at the cell surface. The cell surface of sea urchin eggs therefore seems to be the locus of active phosphorylation.

The experiments reported in the present paper show that UN blocks the cortical change of sea urchin eggs induced by spermatozoa or butyric acid, whether the eggs are subjected to the activation procedures subsequent to pretreatment with UN or in presence of UN. If UN really acts on the cell surface of sea urchin eggs as it does in yeast, this may lend support to the hypothesis presented in the preceding paper of this series, that high energy phosphate compounds may play some role in the cortical change of sea urchin egg. It was concluded previously that the point of participation of high energy phosphate in the chain of reactions underlying the cortical change may be one of the early steps which precedes the visible changes [6]. This conclusion was mainly based on the fact that DNP and NaN_3 block the cortical change produced by spermatozoa, butyric acid or urea by way of the fertilization wave, but do not inhibit that caused by Monogen and sodium choleinate, which may act on cortical granules more directly without the intervention of the fertilization wave [11]. According to the present results, the same holds true for the inhibitory action of UN-pretreatment and the presence of UN. It is therefore probable that the same step in the chain of reactions underlying the cortical change is blocked directly by DNP, NaN_3 and UN, and that in this step a phosphate-involving reaction is included. The possibility that this reaction is the fertilization wave was suggested in a previous paper [6]. Several experiments reported in the present paper concerning the effect of the presence of UN on the cortical change induced by butyric acid may provide further material for the discussion of this issue.

The fact that the cortical change was blocked by exposing the eggs to UN-sea water only 12–20 seconds before the moment at which the membrane elevation would start seems to indicate that UN acts extremely rapidly. It may also be seen from the data that UN is easily removed by washing. Admitting an almost instantaneous action of UN and its quick removal by washing, the following conclusions were deduced from the data concerning the effect of UN on the various phases of butyric acid activation procedure. (a) UN acts on some reaction taking place mainly in the second step of butyric acid activation procedure (while the eggs are in sea water following the exposure to butyric acid-sea water); (b) the UN sensitive reaction is, however, not directly involved in the visible changes, but is a reaction that precedes them. The first conclusion, however, does not rule out the possibility that the UN-sensitive reaction takes place also during the first step treatment. One of the most plausible explanations is that the UN-sensitive reaction starts during the first step treatment, i.e. in butyric acid-sea water, and takes place also during the first half of the period between the beginning of the second step treatment and the starting of the membrane elevation.

In a recent experiment, Sugiyama stretched the egg in the glass capillary and exposed it to butyric acid sea water at one of the free surfaces. After a considerable lag period the granule breakdown was observed in the unexposed surface [12]. From this observation it appears that the fertilization wave is able to take place in the presence of butyric acid. However, it remains to be decided at what step of the ordinary procedure of butyric acid activation the fertilization wave does occur. It is therefore not impossible that the fertilization wave, although it constitutes one of the early steps of the chain reaction, mainly takes place subsequent to the transfer of the egg into normal sea water. Further experimental tests are necessary to determine whether UN-sensitive reaction is actually the fertilization wave.

Thus it may be suggested that an early step of the cortical change (which might be the fertilization wave) is underlain by some phosphate-involving reaction on the cell surface, and that UN blocks it by forming a complex with the phosphate compound involved. In this connection it is important that $Ba^{\cdot\cdot}$, which also forms complexes with certain phosphate compounds, behaves like UN in inhibiting the cortical change. Moreover, it was shown by Rothstein and Meier [9] that among a variety of cations tested $Ba^{\cdot\cdot}$ and $Zn^{\cdot\cdot}$ most strongly compete with UN for the yeast surface loci. $Zn^{\cdot\cdot}$ is also known to inhibit the cortical change of sea urchin eggs at low concentrations [3].

Concerning the effect of pretreatment with UN, the following might be added: Exposure of the eggs to UN prior to the activation treatment may, like the pretreatment with DNP and NaN_3, inhibit the basal phosphate metabolism, e.g. oxidative phosphorylation of the cortex, thereby indirectly influencing the phosphate including reaction involved in the cortical change.

SUMMARY

1. Pretreatment of unfertilized sea urchin eggs with UN (10^{-4}–$5 \times 10^{-4}\,M$, pH 5.5–7.0) inhibits the cortical change induced by spermatozoa or butyric acid, while the cortical change caused by Monogen or sodium choleinate is not inhibited.

2. If eggs are fertilized in the presence of UN, the cortical change is impaired to a certain extent.

3. The presence of UN inhibits also the cortical change induced by butyric acid. In order to determine what step of the chain of reactions making up the cortical change is directly blocked by UN, one or both of the procedures (exposure of eggs to butyric acid and subsequent washing with normal sea water) were carried out in the presence of UN. It was shown that UN acts on some reaction which takes place mainly after the transfer of the eggs to normal sea water.

4. In the presence of UN, occasionally a pronounced delay of the membrane elevation (taking place 3–15 minutes after butyric acid treatment) was observed.

5. When eggs exposed to butyric acid-sea water in the presence or absence of UN are put into UN-sea water and then transferred to normal sea water, the membrane elevation takes place, unless the duration of exposure to UN exceeds a certain limit (30–120 seconds). Within this limit, membrane elevation is delayed and increasingly impaired as the duration of exposure to UN-sea water is prolonged.

6. Eggs treated with UN-butyric acid-sea water were put into normal sea water and then transferred to UN-sea water. The results indicate that the UN-sensitive reaction mainly takes place in the first half of the period between the transfer of eggs from butyric acid to normal sea water and the beginning of the membrane elevation.

7. The cortical change induced by Monogen and sodium choleinate is not at all inhibited by UN.

8. $Ba^{\cdot\cdot}$ also blocks the cortical change induced by spermatozoa and butyric acid.

9. The possibility is discussed that one of the early steps of the cortical change, which might be the fertilization wave, is underlain by a phosphate-involving reaction on the cell surface, and that UN blocks it by forming complexes with such phosphate compounds.

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THE STRUCTURE AND FORMATION OF PIGMENT GRANULES IN HUMAN HAIR

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The information obtained by light microscopy concerning the pigmentary system of the mammalian epidermis has been summarised recently by Medawar [2, 10] and by Masson [8]. The granular pigment found in the epidermal cells is formed exclusively by melanocytes, dendritic cells found scattered among the germinal cells of the skin and those of the hair follicle, which after forming the pigment granules, pass them along their arborescent processes into the cells of the Malpighian system.

The pigment granules themselves, though varying in size from species to species, are commonly of the order of a μ in diameter. The study of the shape and structure of so small a particle is aided by the higher resolving power of the electron microscope and several papers have appeared describing the appearance of isolated granules in this instrument [1, 4, 5, 6].

The present paper is concerned with the internal structure and the formation of pigment granules in the human hair follicle and forms the sequel to a previous paper [1].

MATERIALS AND METHODS

Ultra-thin sections both of chemically isolated granules and of hair roots from the human head were examined electron microscopically. Several samples of hair were used, but all were light to dark brown when seen in mass and from white European males.

Preparation of sections of hair roots.—A fuller description of the treatment of plucked roots will be found in the earlier publication [1]. Briefly, hairs were plucked and the root immediately immersed in a 1 per cent osmium tetroxide solution buffered at pH 7.3 and cooled to about 5°C [11]. The material was allowed to fix for 1–2 hours, then dehydrated in a succession of alcohols of increasing strength and finally embedded in a methyl-butyl methacrylate polymer according to the now standard procedure. The sections were cut using a modified Cambridge rocker microtome [3].

Preparation of sections of pigment granules.—Pigment granules were isolated from a single sample of dark brown hair by three methods: (*a*) The hair was heated at 100°C for 15 minutes in $N/1$ KOH. This treatment digested the keratin and left a suspension of melanin pigment granules which were centrifuged off and washed with several changes of water. (*b*) The hair was digested at 40°C in a $4M$ solution of urea containing 1 per cent papain and 2 per cent sodium metabisulphite adjusted to pH 7 with alkali [7]. The hairs were almost completely disintegrated in a few hours and, on centrifuging the solution, the pigment granules and some resistant remnants of keratin were obtained and washed as under (*a*). (*c*) The hair was refluxed with $3N$ HCl for 12 hours. The residue was washed as before.

To embed the granules, they were first dried in absolute alcohol (24 hours) and then suspended in monomer. Small plugs were made by centrifuging the suspension in monomer, portions of these were then transferred to monomer containing the catalyst (benzoyl peroxide 1 per cent) and polymerised at 40°C.

DISCUSSION OF THE ELECTRON MICROGRAPHS

Structure of the granules.—Intact granules examined electron microscopically without sectioning appear usually as dense, elongated bodies in which little internal structure can be made out [1, 6]. Sectional material (200–500 Å in thickness) either of isolated granules or of granules in melanocytes, however, permits some definite conclusions to be drawn.

Chemically degraded granules often reveal signs of an internal structure. Furthermore, it is not uncommon for granules to shatter when sectioned and, assuming that rupture may occur along lines of some structural significance, this can sometimes be turned to good account. Most of the granules appear to be composed of numerous small irregular particles, a finding to which little of significance can be attached. Two patterns seem of more consequence: in one the fragments form a series of concentric shells more or less completely developed; in the other several parallel, straight lamellae appear. In Fig. 2, which is an example of a section of granules isolated and partly degraded by alkaline digestion, such structures may be noted. These also appeared after acid digestion, but it was observed that alkali was much more effective in destroying the granules than was acid. After 12 hours heating in $3N$ HCl at 100°C the granules were less broken down than those illustrated in Fig. 2 (15 mins. in $N/1$ KOH at 100°C) and were still held by a tenuous resistant residue in orientated strings with the long axis of the granule parallel to the original fibre axis.

The sections of granules isolated by the enzymatic digestion method were more homogenous and revealed few indications of internal structure.

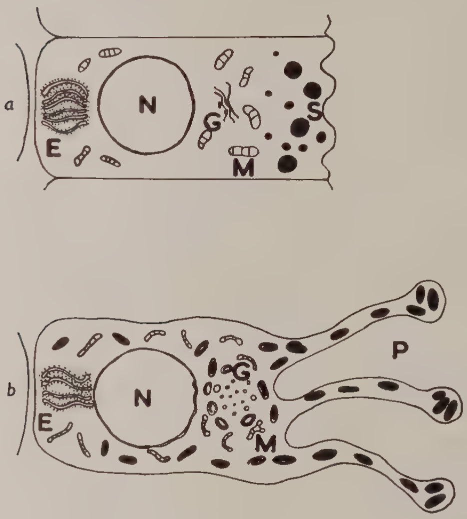
These findings show that many pigment granules possess an internal structure in the form of parallel lamellae, which may round and join at the ends to form more or less completed cylindrical or ellipsoidal shells, or may remain open. Some of these formations suggest that the granule may have grown as a concretion within a small enclosure. Since this structure can be seen best in granules within melanocytes, i.e. presumably incompletely hardened forms, and in those subjected to the action of boiling alkaline or acid solution, it may be inferred that the dense particles of the granule (melanin?) are cemented together by a different substance (protein?) removable by strong reagents.

The melanocyte and granule formation.—In sectioned material from the hair follicle both melanocytes and keratin forming cells may be recognised and have been described by Barnicot *et al.* [1].

The cytoplasms of the two kinds of cells are distinguished in other ways. The melanocyte, like most secretory cells was found to contain a compact cluster of ergastoplasmic membranes usually proximal to the papilla and many mitochondria mostly located immediately distal to the nucleus as shown in Fig. 3 and in the diagram Fig. 1. More characteristic of melanocytes are the vesicles (*V* in Figs. 4 and 5) ranging in diameter from a few hundred Å to about 1 μ which crowd among the mitochondria in the same region. A halo of pigment granules surrounds and defines this region, which centrally is rather free from granules. Granules in various stages of completion were found in many of the larger vesicles (see Fig. 5) suggesting that these are the sites of accumulation of the dense pigment. In a geometrical sense this region of the cytoplasm richly populated with vesicles corresponds to the Golgi regions of other cells. These relations have been indicated in Fig. 1.

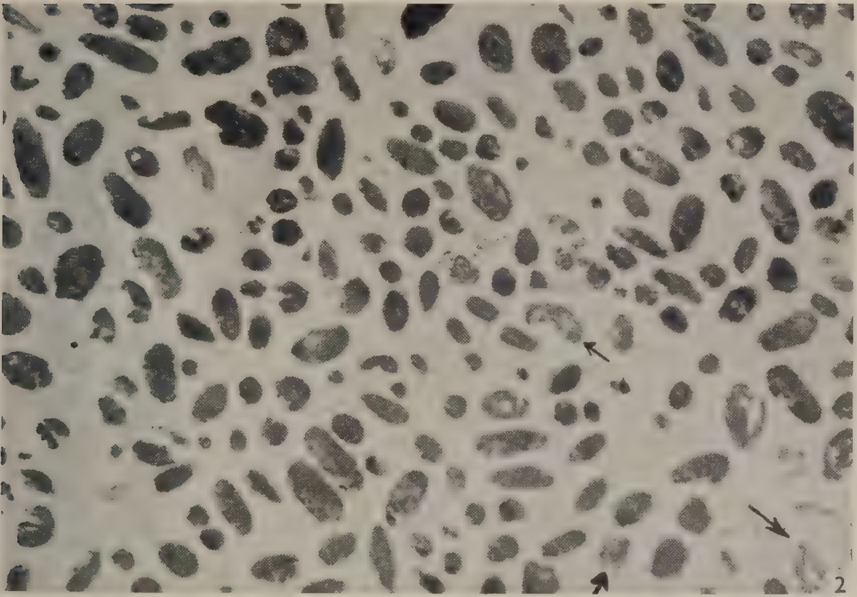
The relation between the melanocyte and the cells of the Malpighian system.—At the level of the hair follicle, where one encounters the nuclei of the melanocytes, the presumptive cortical cells of the hair have a very generalised appearance [1, 14] containing no pigment, few keratin fibrils and large numbers of dense, free particles ca 200 Å in diameter (Palade particles). The melanocytes are usually separated from these cells by a well marked space, which may be accentuated by fixation, but which, in any case, seems to point to a lack of adhesion of the cells of the two systems compatible with the somewhat mobile role one supposes the melanocyte plays.

One or two cell diameters higher up the follicle one finds elongating presumptive cortical cells and between them slender processes of the melanocytes densely packed with granules. At this level some cortical cells contain pigment granules and one occasionally encounters a cell containing a discrete bundle of granules (Fig. 6). As far as we can see these bundles (circular in section and about 2–3μ wide) are contained within the cytoplasm of the cortical cell. Two membranes surround some pigment bundles (one from each cell?); in others these have in part disappeared. Further the membranes of the Malpighian cells at this level of the follicles are often thrown into folds which seem at times to be enveloping the melanocyte process. These appearances are at least compatible with the idea that the cell tip has been phagocytosed and the granules released after digestion of the cell membrane. The pigment bundles disperse higher in the follicle where the pigment comes to lie in strings between the growing fibrils of keratin with their long axis parallel to the fibre axis. When isolated from hair by acid digestion of the keratin similar strings of oriented granules held together in a tenuous resistant matrix are obtained.



Text-fig. 1. A comparison of the organisation of a typical secretory cell (*a*) possessing polarity and that of a melanocyte (*b*). *E* is the ergastoplasm, *N* the nucleus, *M* mitochondria, *G* the Golgi region, and *S* the secretion. In the melanocyte a similar polarity appears. *G* denotes the region in which the pigment granules appear and *V* the melanogenic vesicles.

The melanocyte processes extending between the keratin forming cells contain, in addition to granules, other formed elements of the cytoplasm and sometimes mitochondria, which are transferred to other cells along with the granules. Such a transfer of potential melanogenic apparatus to a receptive cell, e.g. a white dentritic cell, could provide a mechanism for the "infective" spread of pigment from pigmented grafts described by Billingham and Medawar [10].



The magnification of the electron micrographs is indicated by a line the length of which is one micron.

Fig. 2. Electron micrograph of a thin cross-section of a plug of pigment granules isolated from hair by alkaline digestion. Many of the granules have ruptured into irregular fragments; a few (indicated by arrows) display the internal structure referred to in the text.

CONCLUSIONS

1. Cross sections of chemically isolated melanin granules and of those found in the melanocytes of the hair follicle show that, while many granules break into irregular small particles, some reveal an internal structure of

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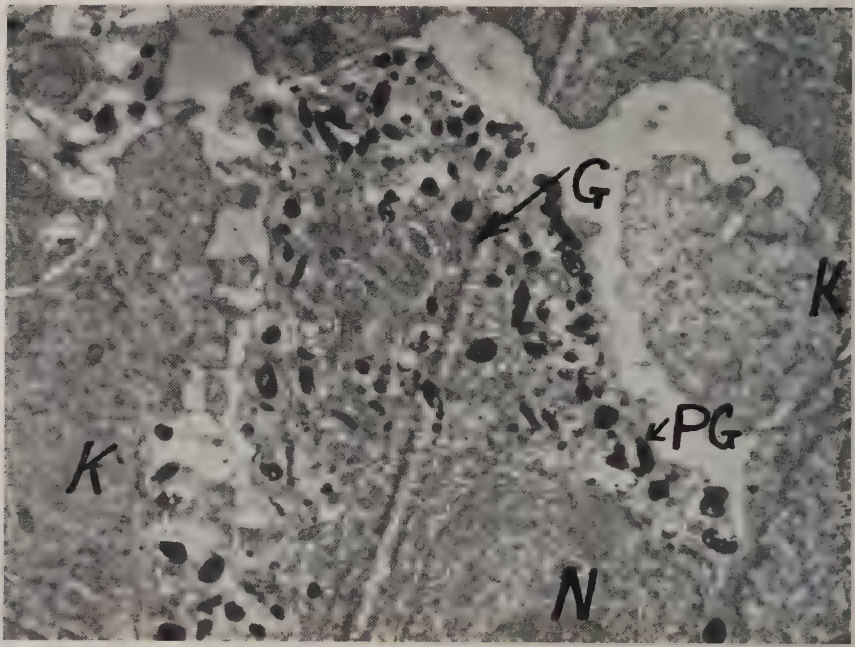
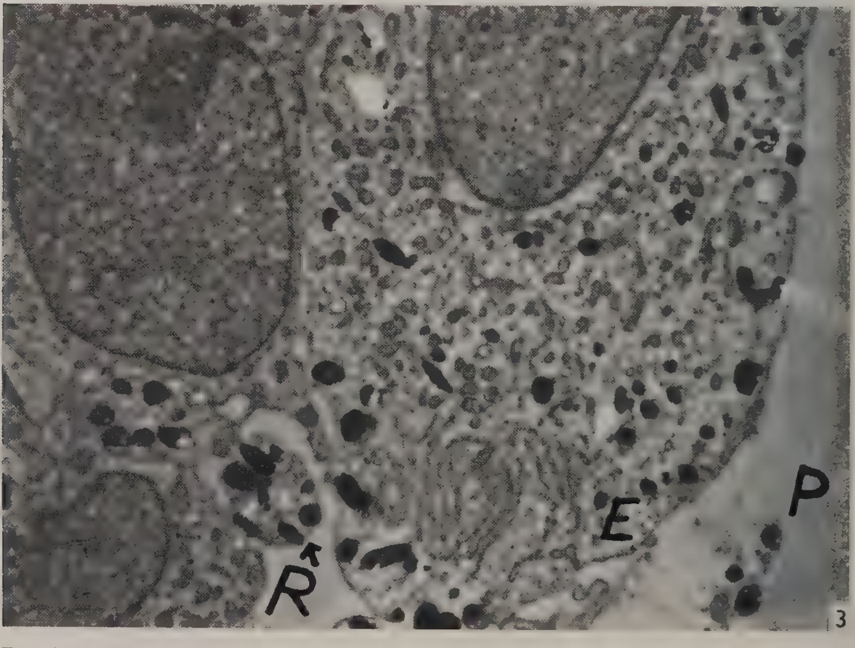
Fig. 3. Low power electron micrograph of portions of two melanocytes and adjacent keratin (Malpighian) cells for the purpose of orientation. *P* is the papillery end of the cell, *E* the ergastoplasmic membranes, *R* a section of a process packed with granules, *N* the nucleus, *G* the vesiculated region, where the pigment accumulates, and *K* a keratin cell.

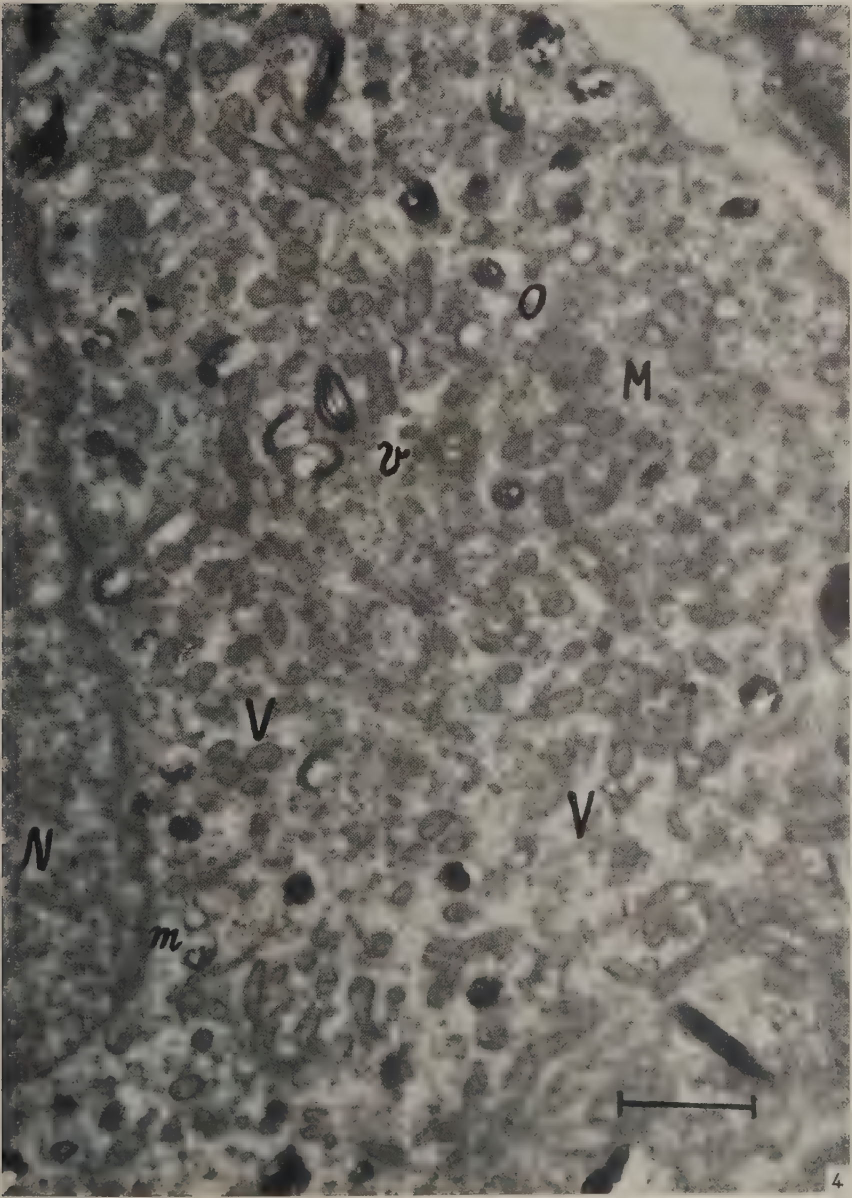
Fig. 4. Section of a melanocyte distal and proximate to the nucleus *N* to show the broad characteristics of the vesiculated region. There are many mitochondria *M* and centrally many vesicles *V*. Melanogenic vesicles are shown at *v*.

Fig. 5. A section of the same region at higher magnification to display the internal features of the melanogenic vesicles. Vesicles containing varying amounts of pigment, which may represent stages in the formation of granules, are indicated by arrows.

Fig. 6. Pigment bundle within a cell of the Malpighian system. Such bundles probably represent tips of the melanocyte processes which have entered the cell. Traces of two surrounding membranes may be seen.


G
K
K
PG
N


E
P
R
3


O
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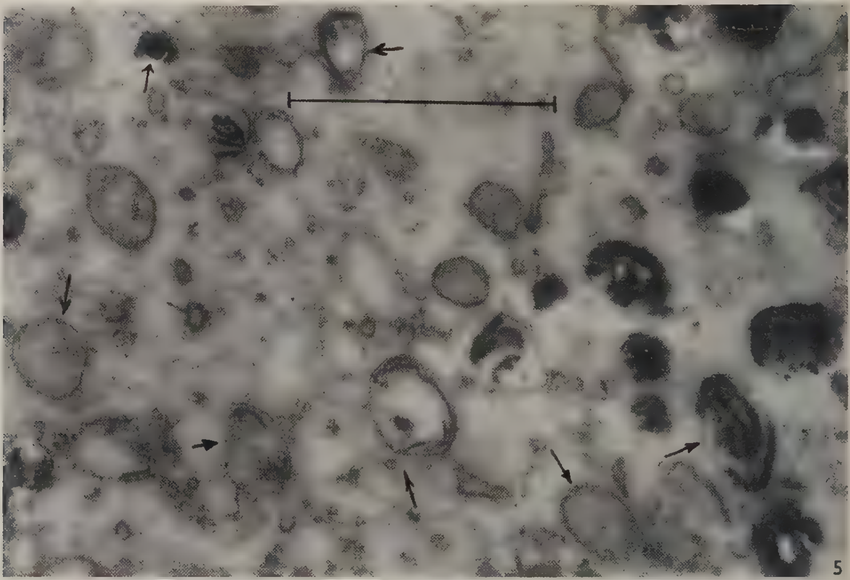

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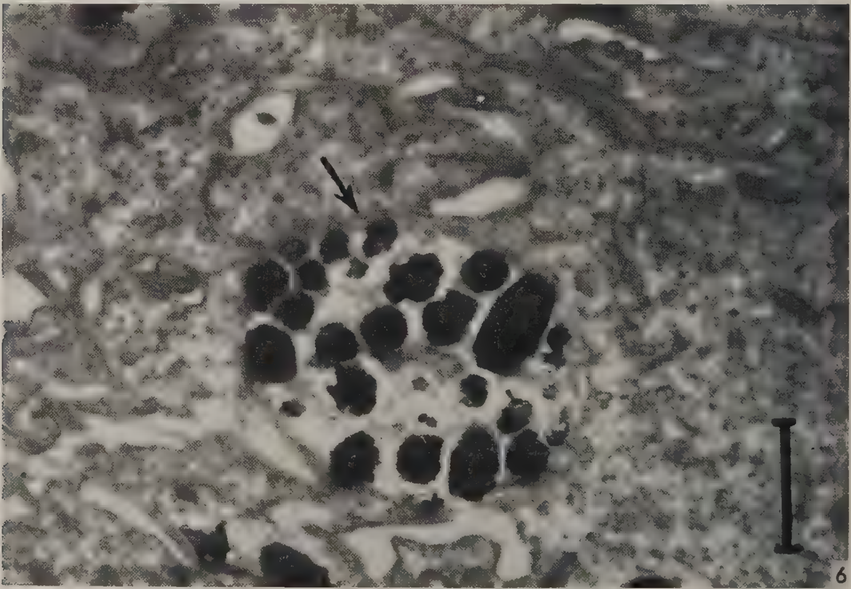

6

parallel lamellae which may either remain open at the ends or round off to form concentric shells.

2. Thin sections of melanocytes in the hair follicle show that these cells have a cytoplasmic organisation resembling in its broad features that of other secretory cells. An endoplasmic reticulum is usually found at the end of the cell nearest the papilla of the hair follicle. A very characteristic region, densely populated with mitochondria and vesicles, but relatively free from pigment granules, lies distal to the nucleus and probably is analagous to the Golgi region of other cells. Surrounding this region are found numbers of partly developed granules in vesicles or vacuoles. These vesicles seem to be the sites of formation of the granules.

3. Fully formed granules have been encountered throughout the cytoplasm. They are most densely packed in the processes where these leave the cell. The processes can be traced as they thread their way between the presumptive cortical cells of the hair. The pigment enters the cortical cells in rounded bundles closely packed with granules which subsequently disperse as the cells elongate and fill with keratin fibrils.

SUMMARY

The preparation of thin sections of melanocytes from the human hair follicle and of pigment granules isolated by various methods from hair is described. Sections of granules viewed electron microscopically suggest they are aggregates of dense particles; some granules appear to consist of several parallel lamellae which may form a succession of cylindrical or ellipsoidal shells.

The pigment granules appear to develop in vesicles occurring in a region distal to the nucleus of the melanocyte probably analogous to the Golgi region of other secretory cells.

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VIRUS ACTIVITY IN THE CYTOPLASM OF ERYTHROLEUKOTIC CHICK CELLS

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The transmissible properties of fowl leukaemia were discovered by Ellerman and Bang in 1908 [1]. In order to understand the pathogenesis of the virus-induced leukaemias and to ascertain which part of the cell is most directly engaged, it is essential to find out the localization of the virus in the cell.

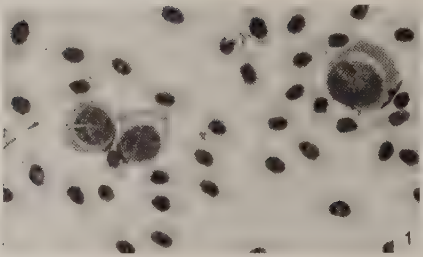
Both the nucleus and the cytoplasm of the leukaemia cell show characteristic cytological and cytochemical changes as compared with normal blood cells [8, 9], and a search for the bulk of inducing activity in either of these constituents is indicated.

In the process of separating the cytoplasm and the nucleus it is impossible to exclude contamination of one fraction with the other. Hence only sufficiently accurate quantitative activity determinations can give information about the distribution of the virus. Since the virus can be identified and assayed only by its capacity to induce leukaemia, animal inoculation must be used for this purpose.

The present paper describes a separation procedure yielding a relatively pure cytoplasmic fraction of the leukaemia cell. The leukaemia-inducing activity of the cytoplasmic fraction is shown by use of a biological titration method to be the same, as, or higher than, the activity of the total disintegrated cells calculated per unit of nitrogen. Some of the results described in this paper have been published in a preliminary form [3, 5, 10, 11].

EXPERIMENTAL

A strain of erythrogenic leukaemia was used (kindly provided by Prof. J. Engelbreth-Holm). This strain has certain advantages as compared with others that were investigated. It gives a very constant pathological-anatomical picture. After intravenous injection of a moderate dose the animals die within 8–14 days, showing a white cell count of about 0.5 mill./mm^3 (Fig. 1) and marked enlargement of the spleen (Fig. 2) and liver; furthermore the bone marrow is largely replaced by proliferating leu-



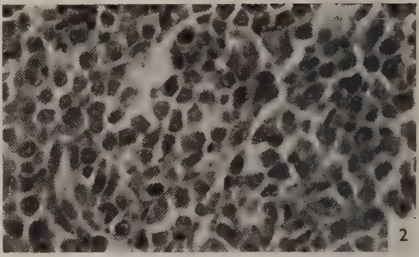


Fig. 1. The erythroleukotic cells in a smear from the peripheral blood. Giemsa stain.
Fig. 2. A section from the enlarged spleen, the parenchyma of which is filled with leukaemic cells.

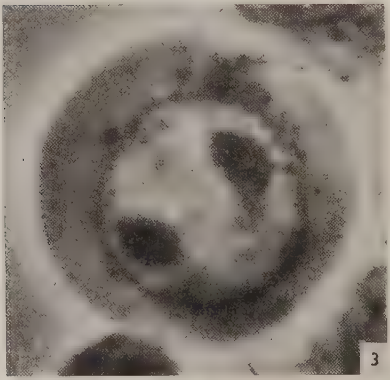
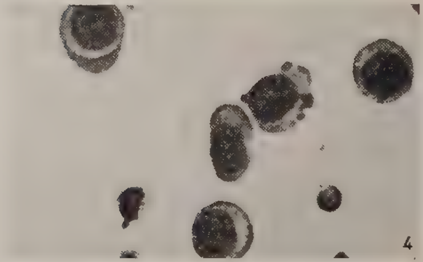


Fig. 3. Phase-contrast picture of a typical leukaemic cell from the strain used in the experiments.



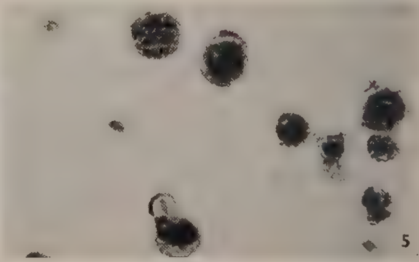


Fig. 4. A smear from a splenic cell suspension. Giemsa stain.
Fig. 5. The same suspension treated in a glass homogenizer. Many cells have lost their cytoplasm.

kaemia cells. The leukaemic cell (Fig. 3) is round with well-defined borders, a markedly basophilic cytoplasm and a round, usually regular nucleus containing one or two big nucleoli embedded in finely dispersed chromatin. Spontaneous leukaemia, in particular of this type, is very rare in Sweden. The control animals never displayed this disease.

An inbred strain of White Leghorn (Edo) chicks was used. This strain has been observed for 12 years, during which time the incidence of "spontaneous" leukaemia (lymphomatosis) was less than 0.1 per cent.

In the earlier experiments 7-day-old chicks were used as a source of cellular material; later 3-week-old birds were used. The animals were injected intravenously with 0.5 ml of a 1 per cent leukaemia spleen tissue extract stored at –90° until the time of injection.

Twelve days later, when the death of some chicks had revealed sucessful infection, all chicks were killed and cell suspensions were prepared from clearly enlarged spleens in the following way: After decapsulation the spleens were finely minced in small pieces, passed through a metal screen and suspended in saline or Tyrode solution. The larger fragments, connective tissue, etc., were eliminated by repeated filtration through sterile gauze. The suspension was centrifuged at 1500 rpm for 4 min. and the white superficial coat pipetted off and resuspended in Tyrode solution. Up to five thorough washings followed. After a final centrifugation the cells were suspended in a smaller amount of Tyrode. Examination with a phase-contrast microscope revealed that very few cells were damaged or clumped together (Fig. 4).

The cell suspension was then treated in a glass homogenizer at moderate speed for 5 min. Phase-contrast examination showed that many cells had lost their cytoplasm (Fig. 5). Counts of the cells and the nuclei made before and after treatment revealed no significant destruction of the latter. Part of the suspension was centrifuged for 10 min. at 2500 rpm. The supernatant was the "cytoplasmic fraction".

The "total cell suspension", containing undestroyed cells, nuclei and the cytoplasmic fraction, was homogenized in a Waring Blendor at high speed for 3 min. with resultant destruction of all cells. Both this and the cytoplasmic fractions were treated in a Waring Blendor to ensure similarity of treatment. The effectiveness of homogenization was checked by phase-contrast microscopy. All preparations were made at +4° C.

The activity of the different fractions.—Titrations were made on the same or the next day; the preparations were invariably stored at +4° C. Series of tenfold dilutions were used. Saline was sometimes used, but in most experiments the diluent was 2 or 10 per cent normal rabbit serum in saline. 0.5 ml of each dilution was injected intravenously into 5- to 9-day-old chicks. Parallel injections of the different preparations were made, the dilutions 10^{-1} from all samples to be tested being first injected, then 10^{-2}, and so on. The positive "takes" were checked by biopsy, when enlargement of the liver and spleen was registered. In dubious cases the reaction was checked by microscopical examination of the liver or spleen.

Table I illustrates the calculation of the activity titre both with the use of the 50 per cent end point, as calculated by Reed and Muench's [6] method, and given as the incubation time index using the incubation time method

[4]. A comparison of incubation time indices of two different materials is possible only if the regression of incubation time (or its reciprocal) upon log. inoculum is the same in the two cases. In most of the present titrations simultaneously made, the regression coefficient for the cytoplasmic fraction was less than the regression coefficient for the corresponding total cell suspension. The mean regression coefficient obtained at 28 titrations including leukaemic spleen extracts, total cell suspensions and cytoplasmic extracts was 0.0186 ± 0.00090. The corresponding figure was 0.019 ± 0.0014 for the total cell suspensions and 0.015 ± 0.0017 for the cytoplasmic fraction. Even though the number of titrations on which these means have been calculated, i.e. 6 in each group, is not large, the results seem to indicate, that there is a probable difference between the regression coefficient for a cytoplasmic fraction and for the corresponding total cell suspension.

TABLE I

Illustrating the course of an experiment by which the virus content may be determined both with the use of the 50 per cent end point and the incubation time method.

Dilution	Days of survival after injection of 0.5 ml diluted leukaemia cell extract into eight-day-old chicks Period of observation 30 days							Leukaemic death of chicks injected
	9	10	11	12	13	14	15	
10^{-1}	1	2	1	2				6/6
10^{-2}		4	1	1				6/6
10^{-3}			2	2		2		6/6
10^{-4}				1	2			3/5
10^{-5}							1	1/6
10^{-6}								0/6

Titre 50 per cent end point	4.3
Incubation time index	6.1
Regression coefficient	0.021

With regard to the causes of this difference no conclusions can as yet be drawn. This point is the object of investigations in progress and will be discussed in a later paper. Until more light has been thrown on this question we do not regard it as advisable to use the incubation time method in comparative determinations of the virus content in total cell suspensions and in the cytoplasmic fraction. It should be emphasized, however, that essentially similar results were obtained when the activity was estimated by the incubation time method and when the titres were calculated according to the 50 per cent end point method of Reed and Muench.

RESULTS AND DISCUSSION

Table II shows the titration values of the cytoplasmic fraction as compared with the titration of the total homogenized cells. The titre of the latter was higher than the titre of the cytoplasm in 3 cases of 6, whilst in the other 3 the titre of the cytoplasm was higher. In no case was the difference more than 1.2 log., which lies within the range of the error of biological titrations of this kind. The mean values of 6 titrations were 3.28 for the total cell suspension and 3.33 for the cytoplasm. The removal of the nuclei would not, then, reduce the virus titre, which seems to indicate that the virus is not localized to this part of the cell. As appears in Table II, the virus content has also been expressed as virus activity per unit of total nitrogen in the titrated material. Similar results were obtained in all experiments, the virus activity of the cytoplasmic fraction invariably being found to be the same as (or higher than) the activity in the total disintegrated cell, as calculated per unit of nitrogen. This also indicates that the bulk of virus is localized to the cytoplasm.

Both the cytoplasmic fraction and the total cell suspension contain non-specific nitrogen. With regard to the total cell suspension nitrogen from the red cells may be the principal contaminant. The red blood corpuscles can never be completely removed and may constitute up to 10 per cent of the original cell suspension. The red corpuscles are not affected by the first homogenization and will therefore exclusively contaminate the total disintegrated cell suspension. By contamination from red cells, not containing virus the virus content of the total cell suspension may be diluted by 10 per cent at the utmost but since the red corpuscles contain only half the amount of nitrogen present in the leukaemic cells (Table III) the relative decrease in

TABLE II

Titre of virus content, 50 per cent end point expressed in decadic logarithms. The activities are calculated per unit of Kjeldahl nitrogen.

Exp. No.	Cells 50 % end point	Cells N mg/ml	Cytoplasm 50 % end point	Cytoplasm N mg/ml	Activity ratio cells/cytoplasm
21	1.57	0.16	0.81	0.02	1 : 1.4
28	3.0	0.42	3.85	0.13	1 : 23
29	2.5	0.44	1.5	0.05	1 : 1
80	2.7	0.70	3.8	0.05	1 : 176
89	3.7	0.46	4.6	0.16	1 : 23
95	6.2	3.40	5.4	0.52	1 : 1

the activity figures cannot be more than 5 per cent. The cytoplasmic fraction may also be contaminated by cytoplasmic nitrogen from nonleukaemic immature spleen cells. These cells (reticulo-endothelial cells, lymphatic elements) are few in number but on the other hand the nitrogen content in the cytoplasmic fraction is low (only $^1/_{20}$ of that of the total cell suspension, cp. Table III); hence even a slight contamination with nitrogen of non-virus origin of this fraction influences the activity figures. No attempt was made to determine the degree of non-specific contamination of the cytoplasm with resultant decrease in the activity figures.

TABLE III

Kjeldahl nitrogen analyses of mixtures between red cell suspension (81,000 cells per mm^3), leukaemia cell suspension (260,000 cells per mm^3) and a cytoplasmic fraction made from the same leukaemia cell suspension. The nitrogen analyses of the different mixtures were made in order to check the method of nitrogen determination.

	N mg/ml	N $10^{-3}\gamma$/cell
Leukaemia cell suspension	2.3	0.009
Cytoplasmic fraction	0.13	—
Red cell suspension	0.33	0.004
$^3/_4$ l.c. + $^1/_4$ r.c.	1.13	—
$^1/_2$ l.c. + $^1/_2$ r.c.	1.07	—
$^3/_4$ cpl + $^1/_4$ r.c.	0.27	—
$^1/_2$ cpl + $^1/_2$ r.c.	0.33	—

The virus is sensitive to oxygen [2]. The final homogenization in a Waring Blendor may result in different degrees of inactivation, due to different viscosities etc., of the two fractions. Table IV shows, however, that under the present experimental conditions the homogenization did not significantly influence the infectivity titre.

TABLE IV

Part of a cytoplasmic fraction (control extract) was kept on ice, another part was treated in a Waring Blendor at high speed for 2 minutes.

Material	10 log. titre 50 % end point
Extract, control	4.0
Extract homogenized in Waring Blendor	4.6

In the original suspension free virus particles may have been absorbed on the surface of the cells. They would then appear in the cytoplasmic fraction without necessarily having been intracellular. In order to elucidate the absorption of virus activity occurring on cell surfaces, a clear and highly active preparation was treated with normal chick erythrocytes. As shown in Table V, only about one thousandth of the original activity remained on the cells after these had been washed twice with amounts of saline comparable to those used in the experiments. Thus surface-bound virus seems to be of minor significance.

TABLE V

1 ml of packed chick red cells was added to 9 ml of a 20 per cent spleen extract. After 2 hours in ice water the cells were spun down, washed twice in 75 ml cold saline and resuspended to the original volume.

Material	10 log. titre 50 % end point
Extract, control	4.8
Red cells exposed to extract and washed	1.8

Further evidence of the intracellular localization of the virus has been obtained by analysis of the cytoplasm and its subfractions. Table VI shows that the erythroleukotic cytoplasm is especially rich in ribonucleotides. If the cytoplasm is further divided on a silica column [7] no greater loss of virus takes place and the activity seems to follow the ribonucleotide fraction very closely. (Some details of this experiment have been published elsewhere [5, 10] and further investigations are in progress). If the possible

TABLE VI

Results of analyses of two cytoplasmic extracts. Nitrogen was determined by the Kjeldahl procedure, phosphorus by a modification of Fiske-Subbarow's method and pentoses by the colorimetric cystein reaction of Brody.

Exp. No.	N mg/ml	P mg/ml	Pentose nucleic acid P mg/ml	Schiff aldehyde reaction	PNAP/P
34	0.0206	0.0040	0.00139	—	35
36	0.0676	0.0085	0.00268	—	32

redistribution of the virus during fractionation is disregarded, its association with the ribonucleotides suggests an intra-cytoplasmic localization.

This does not necessarily mean that the virus is composed of ribonucleotides. As can easily be calculated, the content of nucleotide molecules in the active fractions is of quite another order of magnitude than the content of active virus particles.

The analytical results of the isolation in bulk of particular cellular constituents have to be reviewed carefully and critically in connection with a discussion of the topographical relationship in the single cell. For details the reader is referred to [9], p. 55. The present experiments seem to indicate, however, that the virus of a fully developed leukaemia cell is mainly located to the cytoplasm. This finding together with the techniques employed provide a new approach to the study of the interplay between the cell and the neoplastic virus metabolism.

SUMMARY

A cytoplasmic fraction has been prepared from erythroleukotic chick cells. Titrations showed that the virus activity of this cytoplasmic fraction was the same as or higher than the activity of the total.

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THE INFLUENCE OF HORMONES ON CELL DIVISION

I. TIME-RESPONSE OF EAR, SEMINAL VESICLE, COAGULATING GLAND AND VENTRAL PROSTATE OF CASTRATE MALE MICE TO A SINGLE INJECTION OF ESTRADIOL BENZOATE

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The mitogenic actions of hormonal materials have been studied in a variety of tissues belonging to the endocrine system proper or to its "target tissues". The results of many such studies have been tabulated by Eigsti and Dustin [14]. Estrogens and androgens have been demonstrated to be powerful mitotic stimulators of both the stromal tissues and epithelia of such glands as the seminal vesicle and ventral prostate [11, 23]. Bullough and van Oordt [10] have demonstrated that these materials are capable of inducing elevated levels of mitotic activity in the malpighian layer (stratum germinativum) of mouse ear epidermis. Dirschel, Zilliken and Dropp [13], in dose-response studies, have shown that a linear relationship exists between dose level and mitotic activity in mouse seminal vesicle when androgens are employed. Estrogenic materials, however, do not yield simple linear curves in this type of experiment. Experiments have been carried out to determine which hormonal factors may act to inhibit mitotic activity. Green and Ghadially [16] have demonstrated inhibition of mitosis in rodent skin following administration of cortisone. Bullough [5] has presented provocative data derived from *in vitro* studies on the effects of estrogen on mitotic activity of ear epidermis which tends to indicate that this material exerts its influence on cell division by intervention in the glycolytic phases of carbohydrate metabolism. In spite of the research effort which has been expended upon the problem of control of mitotic activity by hormonal materials, no clear picture of the points of hormonal interposition or of the hormonal interrelationships involved has as yet emerged.

Much of the work concerned with the influence of hormones upon cell division has had as its goal the simple demonstration of the fact that a particular hormone is capable of inducing mitotic activity in a given tissue [22]. Data are often presented in relative terms such as the number of mitotic figures per microscopic field [11] thus making comparison of levels of activity reached by tissues of different cell densities difficult or impossible. Ignorance of the existence of diurnal mitotic rhytms [8] and age factors [7], a lack of appreciation of the proper use of the colchicine technique [6], and absence of control over genetic variability in test animals serve to vitiate much of this work. Burkhart's paper [11] on the early effects of androgenic materials appears to stand alone as a detailed time-response study in this area of investigation. A preliminary study on the time responses of ear and seminal vesicle to estrogenic materials was reported by Allen [1].

The need for time-response data derived from experiments in which intrinsic and extrinsic variability is controlled as closely as possible is apparent. The present paper, the first of a series of time-response studies, deals with the development of mitotic activity in the epithelial cells of ear epidermis, seminal vesicle, coagulating gland and ventral prostate under the influence of a small dose of estradiol benzoate.

MATERIAL AND METHODS

All work was carried out using 90–180 day old, purebred BALB/c Jax castrate male mice. Castration was performed 30 days prior to use of animals for experimental purposes. The experimental stock was housed under conditions of constant 14 hour artificial day length (0700 hours to 2100 hours). A standard laboratory ration and water were provided *ad libitum*. Estradiol benzoate (Scheering) was administered as a single subcutaneous injection of 16 micrograms (100 Rat Units) in .25 cc sesame oil (N.F.). Colchicine (Penick, powdered U.S.P.) was utilized to arrest mitoses and was administered as a single subcutaneous injection of 100 micrograms in .25 cc distilled water 6 hours prior to autopsy time. Solutions of colchicine were routinely made up just prior to use. Autopsy was carried out at 1400 hours. Hormone and

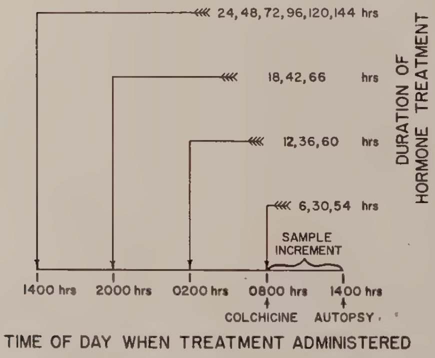

Fig. 1. Plan of experimental time relations employed to allow harvesting of mitoses during a fixed time period. Horizontal axis indicates time of day when treatments were given, arrows show time of administration of hormone for a given duration of treatment (right vertical axis).

colchicine administration were adjusted to this autopsy time as indicated in Fig. 1. Six hour samples of mitotic activity were thus made available from 0 hours to 72 hours following hormone treatment and further 6 hour samples covered the periods following hormone treatment of 90–96 hours, 114–120 hours, and 138–144 hours. Castrate controls received identical treatment save for omission of estradiol benzoate. This experimental procedure serves to fix all experimental variables. Animals were killed by cervical dislocation and subjected to immediate autopsy. Seminal vesicle, coagulating gland, ventral prostate, and samples of pinna were dissected out, fixed in Bouin's fluid, subjected to standard histological processing procedures, serially sectioned at 8 microns, stained in Harris haematoxylin and eosin and mounted. For analysis of mitotic acitivity resting nuclei versus colchicine arrested methaphases of the acinar epithelial cells of ventral prostate, coagulating gland and seminal vesicle and the epithelial cells of the malpighian layer of pinna were differentially counted. Three thousand non-dividing cells were observed in each tissue in each animal and 5 animals constituted a typical experimental group. Counting was carried out at 440 diameters magnification using an ocular counting grid. To prevent prejudicing of counts, slides were randomized and counted as unknowns. Raw counts were converted to per mille mitotic indices, the per mille indices multiplied by 100 and converted to logarithms. From these figures standard errors of the means and 95 per cent confidence limits were calculated using standard statistical methods. Zero values were corrected for discontinuity by dividing the number of observed cells into 250.

RESULTS

Comparison of mitotic indices exhibited by the four investigated tissues in animals with intact testes as opposed to castrate control animals (Table I) indicates that all tissues show a decrease in mitotic activity following castration. Using t-tests this decrease is shown to be significant in all cases ($P = < .02$).

The response of ear epidermis to hormone injection in castrate males is presented graphically in Fig. 2. Inspection of this figure indicates that ear epidermis undergoes a pronounced drop in mitotic activity during the 6 hour period (0–6 hours) immediately following administration of estradiol benzoate. The t-test indicates this drop to be significant ($P = < .02$). A significant increase in mitotic activity over castrate levels is noted in the period 6–12 hours following hormone administration ($P = < .05$). Peak activity is reached about 24 hours after estrogen treatment. Any lag period preceding commencement of mitotic activity in this tissue is obscured by the above mentioned depression following estrogenization. Inspection of the "plateau" portion of the curve shows a series of regular fluctuations to be present which have an apparent period of 24 hours. Analysis of variance indicated that these points form a heterogeneous series ($P = < .001$ between 12–72 hours) thus suggest-

TABLE I

Mitotic activity at six hour intervals following the injection of 16 micrograms estradiol benzoate into castrate male mice. For each tissue mean mitotic indices as log (per mille mitotic index × 100) followed by standard error values are given. The number of donor mice for tissues examined follows in brackets.

Sample category	Ear	Seminal vesicle	Coagulating gland	Ventral prostate
Normal	2.954 ± .031 (5)	1.620 ± .222 (5)	1.899 ± .062 (5)	2.220 ± .049 (5)
Castrate	2.644 ± .073 (4)	1.032 ± .119 (5)	1.038 ± .154 (4)	1.056 ± .023 (4)
0–6 hrs.	2.377 ± .070 (4)	1.027 ± .156 (4)	.898 ± .006 (5)	
6–12	2.902 ± .045 (5)	1.388 ± .281 (5)	.896 ± .029 (5)	
12–18	3.014 ± .075 (5)	1.390 ± .233 (5)	1.149 ± .151 (5)	
18–24	3.085 ± .013 (5)	1.846 ± .158 (5)	1.143 ± .143 (5)	1.151 ± .148 (5)
24–30	2.973 ± .034 (4)	2.607 ± .029 (5)	3.326 ± .078 (5)	
30–36	2.943 ± .117 (5)	3.110 ± .080 (5)	3.218 ± .119 (5)	
36–42	3.004 ± .084 (5)	3.043 ± .036 (5)	3.578 ± .048 (5)	
42–48	3.097 ± .060 (5)	3.019 ± .049 (5)	3.183 ± .029 (5)	1.279 ± .148 (5)
48–54	2.820 ± .063 (5)	3.269 ± .025 (5)	3.283 ± .038 (5)	
54–60	2.809 ± .023 (5)	2.941 ± .076 (5)	3.112 ± .069 (5)	
60–66	2.908 ± .062 (5)	3.100 ± .075 (5)	3.177 ± .062 (5)	
66–72	2.974 ± .039 (5)	3.188 ± .019 (5)	3.012 ± .048 (5)	1.635 ± .072 (5)
90–96	2.715 ± .064 (5)	2.708 ± .081 (5)	2.580 ± .018 (5)	
114–120	2.650 ± .025 (5)	2.596 ± .048 (5)	2.486 ± .064 (5)	
138–144	2.635 ± .052 (5)	2.595 ± .049 (5)	2.554 ± .088 (5)	1.312 ± .206 (3)

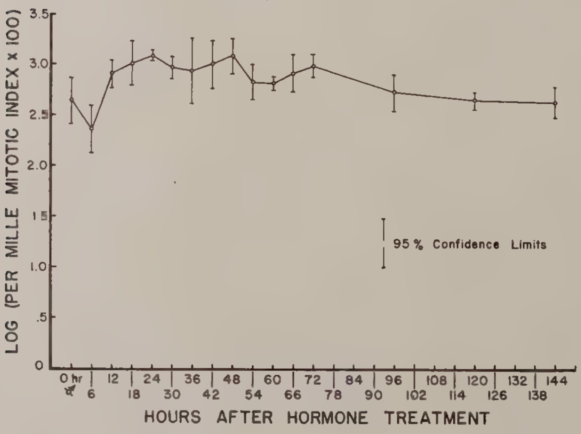

Fig. 2. Time-response of ear malphigian layer epithelial cells to a single injection of 16 micrograms of estradiol benzoate. Ninety-five per cent confidence limits are indicated by vertical lines. Each point on the abscissa (X) indicates the six hour period during which mitoses were arrested by colchicine and the actual time period involved is $(X-6)$ to X hours.

ing the fluctuation to be real. Mitotic activity has returned to castrate levels 96 hours following administration of hormone.

Data obtained for seminal vesicle are represented graphically in Fig. 3. A significant increase in mitotic rate above castrate levels is noted during the period 18–24 hours following hormone administration ($P = < .001$). Peak activity is reached about 36 hours following hormone administration. The "plateau" portion of the curve presents a series of regular fluctuations which have an apparent period of 18 hours. Analysis of variance indicated that these points form a heterogenous series ($P = < .01$) thus suggesting the presence of real periodicity in the data. Mitotic activity is depressed after 72 hours but is still significantly greater (by inspection) than castrate levels 144 hours following hormone administration.

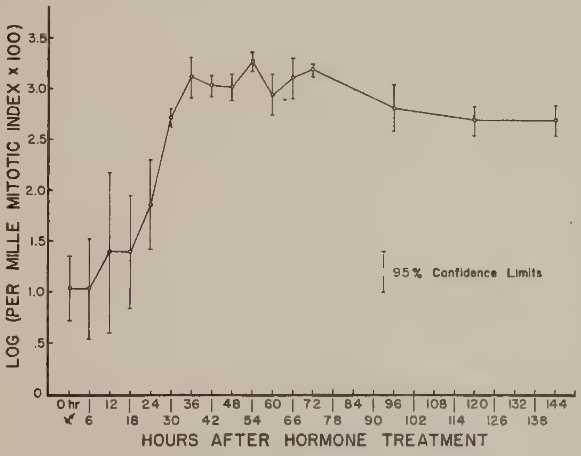

Fig. 3. Time-response of seminal vesicle epithelial cells to a single injection of 16 micrograms of estradiol benzoate. Ninety-five per cent confidence limits are indicated by vertical lines. Each point on the abscissa (X) indicates the six hour period during which mitoses were arrested by colchicine and the actual time period involved is ($X - 6$) to X hours.

Mitotic activity exhibited by coagulating gland is presented graphically in Fig. 4. The first significant increase in mitotic activity over castrate levels occurs during the period 24–30 hours following administration of estradiol benzoate ($P = < .001$). Peak activity appears to be reached at about 42 hours. Inspection of the "plateau" portion of the curve indicates periodic fluctuations at 12 hour intervals. Analysis of variance indicate these points to form a heterogenous series ($P = < .01$) and suggests the fluctuations to be real. Mitotic activity is depressed 96 hours following hormone injection but is still significantly higher than castrate levels (by inspection) 144 hours after treatment.

Ventral prostate response is represented graphically in Fig. 5. Samples were taken only for the periods 18–24, 42–48, 66–72 and 138–144 hours following hormone treatment. It will be noted that only slight increases in mitotic activity are exhibited by the tissue following estrogen administration. Only during the period 66–72 hours after hormone treatment is an increase in mitotic activity above castrate levels noted ($P = < .001$).

Fig. 5 also shows data for all tissues examined plotted on the same set of coordinates. Examination of the data presented in this fashion makes evident

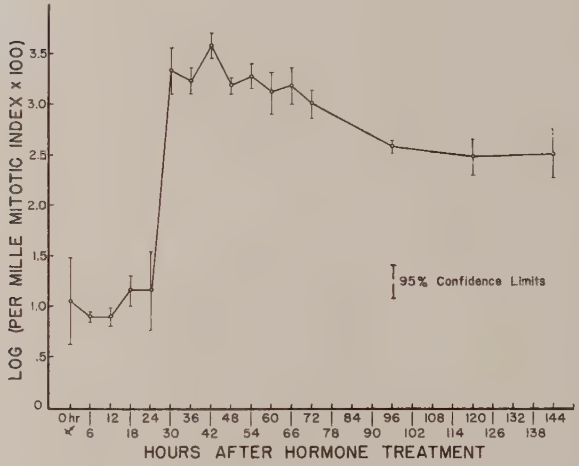

Fig. 4. Time-response of coagulating gland epithelial cells to a single injection of 16 micrograms of estradiol benzoate. Ninety-five per cent confidence limits are indicated by vertical lines. Each point on the abscissa (X) indicates the six hour period during which mitoses were arrested by colchicine and the actual time period involved is ($X-6$) to X hours.

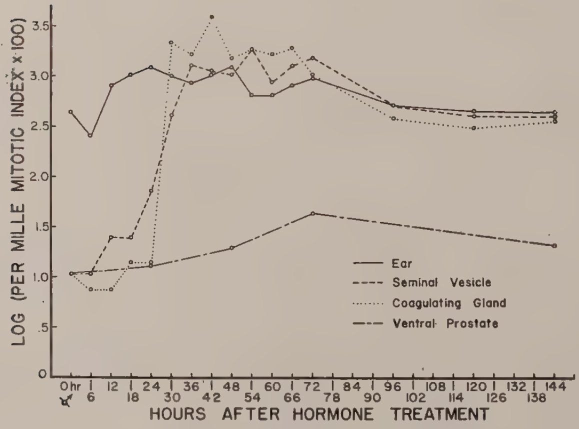

Fig. 5. Time-response of the epithelial cells of malpighian layer of ear, acinar epithelial cells of seminal vesicle, coagulating gland and ventral prostate to a single injection of 16 micrograms of estradiol benzoate. Each point on the abscissa (X) indicates the six hour period during which mitoses were arrested by colchicine and the actual time period involved is ($X-6$) to X hours.

several further points. (*a*) All four tissues show a lag period before commencement of elevated mitotic activity occurs. The lag period for ear is 6 hours, for seminal vesicle 18 hours, for coagulating gland 24 hours and for ventral prostate at least 48 hours. (*b*) The rates at which the tissues reach the "plateau" portions of their curves differ. The rate, expressed as the average per cent of increase above castrate levels per hour, for ear epidermis is 22 per cent/hour, for seminal vesicle 17 per cent/hour and for coagulating gland 131 per cent/hour. (*c*) Although curves for ear, seminal vesicle and coagulating gland reach apparently comparable levels during the period 36–72 hours following hormone treatment, comparison of the mean of means indicates that the average level reached by seminal vesicle is greater than that reached by ear epidermis ($P = < .05$) and that coagulating gland reaches higher average levels than does seminal vesicle ($P = < .01$). (*d*) In terms of relative levels of activity, seminal vesicle and coagulating gland are far more active in their response to estrogen than is ear epidermis.

DISCUSSION

Bullough and van Oordt [10] found that castration lowers mitotic activity in ear epidermis of male mice and that estrogen treatment of castrate males raises this activity. The results reported here confirm and quantify these observations and extend them to the epithelia of seminal vesicle, coagulating gland and ventral prostate of castrate male mice.

The depression of mitotic activity observed in ear epidermis immediately following administration of estradiol benzoate may be due to the action of hormones of adrenal origin released in response to the injected estrogen. Gemzell [15] has demonstrated an increase in the formation and liberation of adrenocorticotrophic hormone in rats following administration of estradiol benzoate, and Green and Ghadially [16] have demonstrated inhibition of epidermal mitosis by cortisone. Inhibition of mitotic activity under the influence of adrenal fraction(s), such as cortisone, may account in part for the lag periods present in the responses of seminal vesicle and coagulating gland to estrogen.

The pronounced difference in time of response to hormonal treatment between ear and seminal vesicle and coagulating gland may be closely linked with the hormone sensitive system(s) through which these materials exert their effect upon cell division. A possible point of hormonal intervention in mitotic activity may be via an effect upon metabolic activity. From a study of the *in vitro* actions of oestrone on ear epidermis Bullough [5] suggests that this material may effect mitotic activity through action on some metabolic step between extracellular glucose and intracellular pyruvate. The hexokinase reaction was strongly favored as the estrogen sensitive rate limiting step. Estrogen has been shown to increase the activity of this enzyme in uterus [26] but biochemical observations on this effect in other target tissues are lacking. *In vitro* studies on the mitogenic action of insulin on ear epidermis [4] indicate a similar influence on the hexokinase system. Studies on the effct of insulin on mitosis in ventral prostate would be of interest in view of the low levels of activity obtained under the conditons of estrogen administration reported here.

A second possible point of hormonal intervention in mitotic activity might involve reactions concerned with the oxidative metabolism of pyruvate. These pathways are, in general, stimulated by androgenic materials and inhibited by estrogens [20]. Bullough and Johnson [9] observed that cell division in ear epidermis is dependent upon the oxidation of pyruvate. The high levels of mitotic activity seen in seminal vesicle and coagulating gland following estrogenization tend to discount reactions involving the oxidation of pyruvate as rate limiting under these circumstances. O'Connor's suggestion [18] that control of cell division may depend upon a balance between the glycolytic and oxidative phases of carbohydrate metabolism is of merit in light of the above discussion.

A third point at which hormonal materials may control mitotic activity is through an effect upon the alkaline phosphatases. Chèvremont and Firket [12] have reviewed evidence which implicates a relation between phosphatase activity and mitotic activity. The action of this enzyme in cell division may be due to (*a*) facilitation of transmembrane glucose transport [21] or (*b*) to its involvement in the synthesis of DNA [12]. Castration depresses the activity of epithelial alkaline phosphatase in rat ventral prostate while estrogen administration restores values to near normal [2]. Since the mitotic response of rat ventral prostate to estrogen [11] is similar to that observed in the mouse, these results indicate that, in this tissue, alkaline phosphatase is not a limiting factor to mitotic activity.

Roberts and Szego [19] followed respiration in rat uterus after intravenous administration of estradiol and found sharply elevated rates of oxygen utilization in this tissue 20 hours following hormone administration. This increase in respiration corresponds with the beginning of cellular proliferation in this tissue. The similarity of this lag period to that observed in seminal vesicle and coagulating gland before onset of increased mitotic activity is of interest.

The disparity in lag phases between seminal vesicle and coagulating gland on the one hand, and ear epidermis on the other, may be explained on the basis of different sensitivities to hormone lack in the two types of tissue. Estrogen, during the 18–24 hours following its administration, initiates the development of metabolic systems to levels compatible with the energy requirements of mitosis in seminal vesicle and coagulating gland. In ear epidermis the metabolic systems are not so absolutely hormone dependent and added estrogen serves only to increase the already high levels of activity still further.

Voutilainen [25] has reviewed the literature dealing with the existence of a 24-hour mitotic rhythm in plant and in normal and malignant animal tissues. He concluded that such a rhythm is a generally observed phenomenon. Bullough [8] found rhythmic activity in the tissues of the mouse to be dependent upon such factors as food intake and body activity. In several tissues he showed similar patterns of diurnal rhythms. Blumenfield [3], to the contrary, found a lack of correspondence between various diurnal rhytms observed in several tissues. If all tissues show a similar 24-hour rhythm of mitotic activity, the "plateau" portions of the curves obtained in the present work would be straight lines or would show random fluctuations since they were sampled at 1400 hours. This is not the case. It must be assumed that these tissues (seminal vesicle, coagulating gland and ear) exhibit rhythms of activity in addition to a regular 24-hour cycle. Two factors may be implicated in this cyclic behavior. (*a*) Exogenously administered estrogen may have variable effects depending upon the condition of the cells upon which it acts. If this be the case, exaggeration of cycles might be expected. (*b*) If the pituitary-adrenal axis is compared to a closed-cycle feed-back system [17, 24], it might be expected that exogenously added estrogen may set periodic oscillations in the liberation of adrenal hormones, which would be reflected as regular fluctuations in the mitotic activity of such tissues as ear and seminal vesicle. The fact that the oscillations observed appear characteristic for each tissue suggests the presence of inherent cellular factors.

SUMMARY

The development of mitotic activity following the administration of a small dose of estradiol benzoate has been followed in the epithelia of ear, seminal vesicle, and coagulating gland of the castrate male mouse. Intrinsic and extrinsic variability have been closely controlled. All tissues respond to estrogen by increased mitotic activity. Seminal vesicle and coagulating gland reach the highest absolute values; ear is intermediate in response; and ventral prostate responds weakly. Ear epidermis responds significantly within 6 hours after hormone administration; seminal vesicle and coagulating gland respond significantly about 24 hours following treatment; and significantly elevated activity is not noted in ventral prostate until 72 hours after hormone treatment. Periodic fluctuations are noted in the mitotic activity curves which suggest the presence of tissue specific rhythms in addition to the previously demonstrated 24-hour mitotic cyle. These results are discussed in terms of the hormonal effects upon the metabolic systems which may be involved in mitotic activity.

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CORTICAL GRANULES IN HAMSTER EGGS

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The elevation of a fertilization membrane as a result of the entry of a spermatozoon into the egg was first described by Fol [5] in sea urchins and starfish. The membrane evidently offers an impassable barrier to other spermatozoa and its elevation has come to be well recognised as an early response to spermatozoon penetration and as one of the protective mechanisms against the penetration of more than one spermatozoon in many invertebrate eggs. The reaction is not specific, however, for it can be evoked by a variety of chemical and physical agents that induce the activation of the egg to further development [8, 18].

Many attempts have been made to explain how the fertilization membrane is formed and Moser [7], observing that certain refractile granules in the cortical region of the vitellus in *Arbacia* eggs disappeared immediately before the elevation of the membrane, put forward a theory that implicated the disappearance of the granules in this process. Runnström and his associates [11, 12, 13] and Endo [4] observed a similar sequence of events and supported Moser's theory in principle, although they differed in their opinions on the actual mechanism involved. Breakdown of cortical granules is probably responsible for the decrease in the amount of light refracted by the egg surface very shortly after entry of the spermatozoon [7].

Cortical granules, in the present connotation, do not appear to have been described in mammalian eggs. This communication is concerned with the occurrence of granules at the surface of the hamster vitellus which seem to be analogous in certain respects to the cortical granules of lower animals.

METHODS

Eggs were recovered from hamsters at various times between one and 18 hr after ovulation and examined with the aid of a phase-contrast microscope.

The artificial activation of eggs was effected by chilling the entire animal to a deep body temperature of about 4° C, for which purpose a procedure similar to that described by Smith, Lovelock and Parkes [15] was employed. This treatment was applied to hamsters in which ovulation had occurred within the previous 12 hr, as indicated by the fact that the vaginal smear consisted almost exclusively of nucleated epithelial cells [17].

Hamsters that were to be killed for eggs undergoing fertilization were kept in a room in which the light was controlled so that ovulation would take place chiefly between midday and 4 p.m. [1]. The animals were kept with males and examined between 11 a.m. and 12 noon each day. The occurrence of mating was deduced from the presence of spermatozoa in the vaginal smear—a true copulation plug was seldom formed.

OBSERVATIONS

Observations on the occurrence of cortical granules were made on 10 oocytes obtained from the ovaries of 3 hamsters and on 218 tubal eggs from 22 hamsters; the data from the tubal eggs are summarised in Table I.

Eggs from unmated hamsters.—Oocytes were obtained from the ovaries of hamsters in which ovulation had taken place within the previous 10 hr.; these oocytes were therefore in stages that were about 4 days before ovulation and probably more, since some oocytes came from follicles devoid of antra. Both the oocytes and the ovulated tubal eggs displayed large numbers of granules distributed more or less evenly over the whole surface of the vitellus (Fig. 1). The density of the granules, as determined in 3 or 4 areas in each of 6 tubal eggs, varied between about 50 and 100 granules per $100\mu^2$. The sizes of individual granules differed but were mostly in the range of 0.1 to 0.5μ.

The granules persisted well *in vitro* during the normal examination period, but diminished in number and finally vanished when, after two or more hours, the eggs deteriorated.

Attempts were made to stain the granules in both living and fixed eggs in a 0.5 per cent solution of Janus green, according to the method of Motomura [9]. In some of the living eggs the granules showed a faint staining but the results were not satisfactory. After fixation with formalin solution or acetic-alcohol the granules could no longer be distinguished, even after the application of the dye.

When living eggs were gently compressed beneath the coverslip and examined by dark-ground illumination a thin bright line was visible around the vitellus (Fig. 3); the appearance suggested that the cortical granules were the light-refracting elements.

In one hamster (No. 2, Table I) 4 eggs were found to have undergone spontaneous activation, as shown by the presence of two polar bodies and a single large, well formed nucleus. In each of these eggs cortical granules were present in the same profusion as in unactivated eggs.

TABLE I

Cortical granules in hamster eggs.

+ + + + = large numbers of granules present.
± = very few or no granules present.

Hamster No.	Treatment	Total eggs	Unpenetrated eggs		Penetrated eggs	
			Unactivated	Activated	Unactivated	Activated
1	None	9	9 + + + +	0	0	0
2		10	6 + + + +	4 + + + +	0	0
3		8	8 + + + +	0	0	0
4		10	10 + + + +	0	0	0
5	Chilled	12	0	12 + + + +	0	0
6		8	0	8 + + + +	0	0
7		12	0	12 + + + +	0	0
8		13	0	13 + + + +	0	0
9	Mated	8	0	0	0	8 ±
10		9	0	0	0	9 ±
11		8	0	0	1 ±	7 ±
12		9	0	0	1 ±	8 ±
13		11	2 + + + +	0	2 ±	7 ±
14		13	11 + + + +	0	0	2 ±
15		9	7 + + + +	0	0	2 ±
16		8	0	0	0	8 ±
17		12	3 + + + +	0	4 ±	5 ±
18		12	0	0	0	12 ±
19		7	2 + + + +	0	1 ±	4 ±
20		8	0	0	0	8 ±
21		12	3 + + + +	0	2 ±	7 ±
22		10	5 + + + +	0	0	5 ±
Totals		218	66 + + + +	49 + + + +	11 ±	92 ±

Four hamsters, killed 3–4 hr. after being chilled to a deep body temperature of 4° C, provided 45 eggs, all of which had undergone activation, having two polar bodies and a single well formed nucleus. All the eggs had cortical granules which, in size, number and distribution, were indistinguishable from those of unactivated eggs.

Eggs from mated hamsters.—Fourteen female hamsters, that had been kept with males and under controlled illumination, were killed between 3.0 and 6.0 p.m. of the day on which spermatozoa were found in the vaginal smear. Of the 136 eggs recovered, 33 had not been penetrated by spermatozoa and were unactivated. All of these had a single polar body, the second maturation spindle in metaphase, and a display of cortical granules similar to that shown by the eggs from unmated hamsters. One hundred and three eggs each contained a spermatozoon, but in 9 of them the spermatozoon had not yet entered the vitellus being still fully within the perivitelline space. In all of these 9 eggs the head of the spermatozoon was touching and apparently attached to the surface of the vitellus, which sometimes showed an elevation at the point of contact. The area of contact usually involved the whole of one side of the spermatozoon head but, in 2 eggs, only the tip of the head touched the vitellus. All the eggs with spermatozoa in the perivitelline space only, had second maturation spindles still in metaphase and one polar body so that they had not yet undergone activation. In the remaining 94 penetrated eggs the spermatozoa had entered the vitellus and the early stages of pronucleus formation and growth were in progress. Two of these eggs, however, had metaphase maturation spindles and so were apparently as yet unactivated although the spermatozoon head had begun its metamorphosis.

In all the penetrated eggs, including those in which the spermatozoon still lay in the perivitelline space, cortical granules were almost completely absent. Sometimes no granules at all could be distinguished in an egg but more often a few could be seen (Fig. 2). When the granules were present their distribution was most irregular: large areas of the vitelline surface would be free of granules but in places approximately 5 to 10 per $100\mu^2$ could be counted. In eggs in which spermatozoon penetration had recently occurred the few remaining granules were found just as frequently near the spermatozoon head (which enters the vitellus first) as in remoter parts of the vitelline surface.

Examination of penetrated eggs by dark-ground illumination showed little evidence of the bright ring about the vitellus observed in unpenetrated eggs (Fig. 3).

DISCUSSION

The cortical granules of hamster eggs have a general resemblance to those described in sea urchin eggs. In particular, the photograph reproduced in

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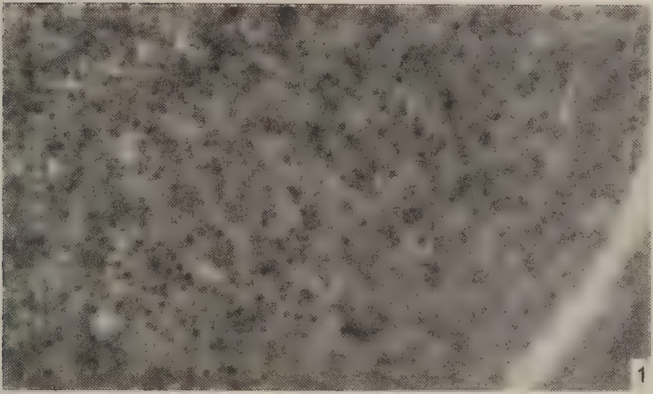
Fig. 1. The cortical granules in a hamster egg before spermatozoon penetration. × ca. 2400.
Fig. 2. The few granules that often remain in parts of the egg cortex after spermatozoon penetration. The head of the spermatozoon is in an early phase of its metamorphosis and lies just under the vitelline surface. × ca. 2400.
Fig. 3. Showing the stronger light refraction by the cortex of an unpenetrated hamster egg (left) as compared to that of a penetrated egg (right). × ca. 130.



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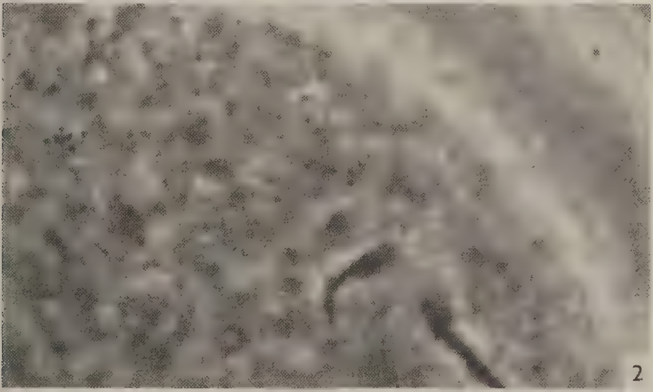

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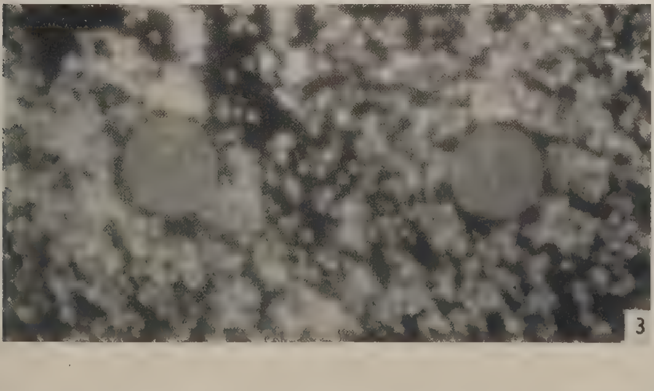

3

Endo's [4] paper, his Fig. 1, which was also taken with the aid of a phase-contrast microscope, is similar to the picture of the hamster egg illustrated in Fig. 1 of the present paper. Endo, however, considers that the sea urchin granules are made up of light and dark halves; the hamster granules appeared to be uniformly dark but they were too small for certainty on this point. The density of cortical granules in the hamster egg is about the same as in sea urchin eggs: 50 to 100 per $100\mu^2$ as compared with 60 in *Clypeaster* [4] and 120 in *Strongylocentrotus* [4, 9]. It has been estimated that there are about 30,000 granules in the whole vitelline surface of the egg of *Psammechinus* [11]: in the hamster the corresponding figure would be in the range of 8000 to 15,000 granules per egg. The hamster granules are evidently a little smaller than those of sea urchins, having a diameter in the region of 0.1 to 0.5μ compared with 0.5 to 1.3μ in the sea urchins [4, 7, 9, 11]. Cortical granules in sea uchin eggs are reported to move out from the inner regions of the vitellus to the surface during the maturation of the egg and to be present there in their final numbers only at the conclusion of the maturation process [9, 10, 11]. Hamster oocytes, on the other hand, which were recovered several days before they would have undergone maturation, had large numbers of granules at the vitelline surface. In this particular the hamster oocyte resembles that of Asterias wherein the cortical granules are present before maturation [11].

Unlike the cortical granules of the sea urchin, those of the hamster could not be stained satisfactorily with Janus green when tested by the methods used by Motomura [9].

The disappearance of the cortical granules in the hamster egg after spermatozoon penetration was not complete but very nearly so. Usually a few granules remained though only in parts of the cortex and even here the numbers were only about 5 to 10 per $100\mu^2$. In the sea urchin, too, the disappearance of granules may not be complete and Moser [7] believed that the granules remaining were of a different character from those involved in membrane elevation. Possibly in the hamster also the residual granules differ in nature from those that respond to the entry of the spermatozoon.

Disappearance of cortical granules in the hamster constitutes a very early reaction on the part of the egg to entry of the spermatozoon; indeed, it may be one of the earliest structural changes for it was evidently elicited by attachment of the spermatozoon head to the surface of the vitellus, even when contact involved only part of the head. Activation of the egg, as recognised by the resumption of the second polar meiosis, is clearly a more delayed reaction for it was not seen before entry of the spermatozoon into the vitellus; in 2 eggs activation had not yet occurred although the head of the penetrating spermatozoon had begun its metamorphosis.

The response of the cortical granules in the hamster appears to be elicited specifically by a stimulus provided by the spermatozoon, since the artificial stimulus of hypothermia, highly effective in inducing activation, had no noticeable action on the cortical granules. This specificity suggests an analogy between the response of the cortical granules in the hamster and the block to polyspermy in the rat, for the block to polyspermy also could not be evoked by procedures that brought about the activation of the egg [2]. It seems improbable, however, that the response of the cortical granules is associated with a block to polyspermy, as the vitelline surface in the hamster is apparently incapable of changing so as to exclude supplementary spermatozoa, protection against polyspermy coming from the operation of the zona reaction [1].

In the invertebrate egg, breakdown of the cortical granule plays a role in the elevation of a fertilization membrane, and it is interesting to note that the presence of a fertilization membrane has twice been reported in the hamster egg [6, 16], in which respect the hamster egg appears to be unique among mammalian ova. These observations, however, were made on fixed material; no sign of a fertilization membrane has been seen in living hamster eggs [1, 3, 14].

It seems possible that the light refraction in the cortex of unpenetrated hamster eggs could be imputed to the cortical granules. Certainly there was much less refraction in the penetrated eggs which had very few cortical granules. A similar change was seen in sea urchin eggs by Moser [7] who also attributed it to the breakdown of the granules. On the other hand, Rothschild and Swann [10] observed a change in the opposite direction, namely an increase in the light-scattering power of the cortex after spermatozoon penetration. This they considered, marked the passage of the "slow component" of the block to polyspermy, but it was probably referable to structural alterations in the cortex, other than granular breakdown.

It is concluded that the disappearance of the cortical granules in the hamster egg is an early and specific response to spermatozoon penetration but that it does not necessarily signify the establishment of a block to polyspermy in the surface of the vitellus.

SUMMARY

The presence is recorded of large numbers of granules at the surface of the vitellus in the hamster egg before spermatozoon penetration. The granules

were mostly between 0.1 and 0.5μ in diameter and occurred at a density of between 50 and 100 per 100μ^2.

Spermatozoon penetration resulted in almost complete disappearance of the cortical granules, and this change took place before the activation of the egg as indicated by the resumption of the second polar meiosis. Activation alone, on the other hand, whether spontaneous or artificially induced, had no noticeable effect on the granules. Disappearance of cortical granules was associated with a distinct decrease in the light-refracting power of the vitelline cortex.

Disappearance of cortical granules in the hamster egg is therefore concluded to be an early and specific response to spermatozoon penetration. It may not, however, be associated with the formation of a block to polyspermy in the surface of the vitellus.

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BRIEF NOTES

THE DEMONSTRATION OF A SECRETORY MATERIAL AND CYCLE IN THE PARENCHYMAL CELLS OF THE MAMMALIAN PINEAL ORGAN

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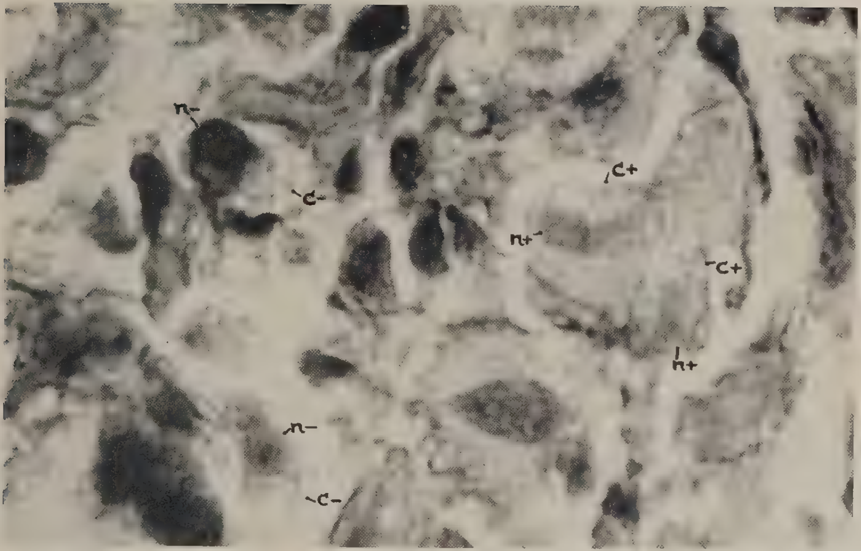
The function of the pineal organ in man and other mammals is still an enigma, although gradually accumulating evidence suggests a relation to antigonadotrophic activity [3]. The state of uncertainty regarding the functional significance of the pineal organ is at least partly due to the lack of evidence clearly demonstrating secretory activity within the pineal. Previously described pineal parenchymal cytoplasmic granulations and staining reactions are not clearly indicative of secretory activity and a cytological secretory cycle has not been successfully demonstrated [1]. A cytological technique for showing the specific activity of pineal parenchymal cells should aid greatly in analyses of the factors regulating the pineal and in the standardization of pineal preparations used in experimental therapy.

It was discovered in the study reported herein that the parenchymal cells of rat (Sprague-Dawley strain) pineals fixed for 16–24 hours in 10 per cent formalin buffered to neutrality with monobasic and dibasic sodium phosphate display an intensive and specific staining reaction with the chrome alum hematoxylin and phloxine technique of Gomori [2] for staining pancreatic islets. After fixation the pineals were washed in distilled water for 24 hours, dehydrated through ethanol solutions, cleared in benzene and embedded in paraffin. Sections seven microns in thickness were then stained according to Gomori's technique [2]. It was found that initial fixation in Bouin's fluid is unsatisfactory and that the step entailing refixation of the deparaffinized sections in Bouin's fluid for 24 hours is necessary for successful results with the pineal. In order to differentiate the cytoplasmic material of the pineal chromophils from the other phloxinophilic structures such as the erythrocytes, the following procedure was added after the phloxine (National Aniline Div., dye content 83 per cent, C.I. No. 778, Certification No. NPh-14) staining, acidification and washing steps in the Gomori procedure: (1) stain for about 1 minute in a solution containing 2 g orange G (National Aniline Div., dye content 93 per cent, C.I. No. 27, Certification No. NO-14), 0.5 g aniline blue (National Aniline Div., C.I. No. 707, Certification No. NK-8) and 1.0 g of phosphomolybdic acid per 100 cc. distilled water; (2) wash rapidly in distilled water and 95 per cent ethanol; (3) dehydrate in two changes of absolute ethanol; and (4) clear in xylene and mount in clarite or HSR. After this procedure, the erythrocytes are colored red and the pineal chromophils are orange or yellow.

Chromophilic and chromophobic parenchymal cells and intermediates are numerous in all rat pineals examined. In the chromophobes, the cytoplasm is clear, the nucleus stained with hematoxylin and the nucleolus stained centrally with phloxine or orange G. In the chromophils the entire cell including the nucleus is stained with phloxine following the chrome alum hematoxylin and phloxine procedure. After staining with orange G and aniline blue the red phloxine-staining of the chromophils is reduced or replaced by a yellow to dark orange staining by orange G. Chromophils in which only the nucleus or only the nucleus and immediately adjacent cytoplasm are stained with phloxine or orange G are common. Chromophobes show a continuous series from small cells with little apparent cytoplasm and small darkly stained nuclei to large cells with a large area of nearly clear cytoplasm around large pale nuclei with large nucleoli. From chromophobes of the latter type, transitional forms are seen to chromophils. The nucleolus enlarges and lies next to the nuclear membrane. Orange areas in the immediately adjacent part of the cytoplasm then appear. Increased amounts of the specifically staining material gradually fill the chromophils, at first, particularly around the nucleus, so that the nucleus is no longer stained with hematoxylin but becomes a blurred dark orange area. The cytoplasm of chromophils, unlike that of chromophobes is usually concentrated in the protoplasmic processes of the cells. The processes, containing intensely stained material, frequently extend to the outer edges of capillaries, which are abundant in the pineal. Occasionally a group of chromophils is found arranged around an irregular area of intensely staining material. Heavily stained chromophils show a non-granular cytoplasm containing scattered, clear vacuoles. The same is true of the material found along capillaries and in clusters of secretory chromophils. Cells that may represent a post-secretory phase of activity have little apparent cytoplasm and a small granular nucleus which still stains with phloxine or orange G. Differential parenchymal cell counts in sixteen rats of both sexes and different ages showed the following ranges in percentages: chromophobes, 41–90 per cent; presecretory chromophils, less than 1–12 per cent; secretory chromophils, less than 1–14 per cent; post-secretory chromophils, 6–41 per cent. It should be noted that while fibroblasts and other cells in the connective tissue can be distinguished from the pineal parenchymal cells in the slides, neuroglial cells, which are much less numerous than the parenchymal cells, are included with the chromophobes since they are not always distinguishable.

The pineal parenchymal cells in the rat, thus, appear to take part in a secretory process. The effects of experimental procedures and the results of histochemical tests on the pineal chromophils will be presented later.

Figs. 1 and 2. Photomicrographs of a small area in a pineal (adult male rat) stained according to the procedure here described. Fig. 1 (top) was taken without the use of filters; Fig. 2 (bottom) was taken with a violet filter at the light source in order to demonstrate the yellow and orange cytoplasmic material of the chromophiles. The nuclear (*n*) and cell (*c*) membranes of two examples each of chromophiles (+) and chromophobes (—) are labeled. Note the presence of intermediate cell types, and the reduction in nuclear size and the obscuring of the nucleus in chromophiles.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AUTORADIOGRAPHIC EVIDENCE OF RADIOSULPHATE UPTAKE BY THYROID GLAND OF RAT

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By means of apposition autoradiography it has been shown that radiosulphate injected into rats is taken up by several organs including thyroid gland [8]. The present communication deals with radiosulphate uptake of thyroid gland studied by electron-track autoradiography, a technique which allows an accurate localization of radioactive compounds in the tissue structures.

Albino rats, weighing approximately 200 g, were given a single subcutaneous injection of $Na_2\,{}^{35}SO_4$ (1.5 μC/g body wt.). 124 hrs. after the injection, rats were killed and the thyroid glands removed, fixed in Carnoy's fluid and embedded in paraffin. Thyroid sections, deparaffinized and hydrated, were coated with Ilford G5 photographic emulsion in "gel form" to a thickness of about 40μ. In some cases the sections were thoroughly washed in water (1 hr.) before being covered with the photographic emulsion. The plates were left for 24 hrs. after the emulsion set and dried, before being processed (temperature development method) [5]. The sections were then stained through the emulsion layer by the Unna-Pappenheim method [7].

Fig. 1 shows that most of ${}^{35}S$ taken up by thyroid gland is localized in the colloid, whilst the epithelial cells exhibit a very weak radioactivity. The same picture has been observed in sections which had been washed for 1 hr. with water before being covered with the photographic emulsion. A comparison with the surrounding tissues (thyroideal and tracheal cartilage, perioesophageal connective, oesophageal epithelium, muscles) showed that the cartilages contain more ${}^{35}S$ than the thyroideal colloid. The radioactivity of the other tissues was negligible.

The question then arises as to the chemical nature of ${}^{35}S$-containing compound which is present in the thyroideal colloid. The fact that this compound is unsoluble in water and in all the solvents used in the histological technique seems to indicate that ${}^{35}S$ is contained in an organic non-lipidic compound. It is likely that ${}^{35}S$ is incorporated into thyroglobulin which is the only component of the colloid still present under these conditions. On the other hand, thyroglobulin is known to contain sulphurated amino acids. However, it has been shown in rats that liver, cartilage and hairs [2, 12] are unable to incorporate radiosulphate into thioaminoacids. ${}^{35}S$ has been found only incorporated into sulphurated mucopolysaccharides in several

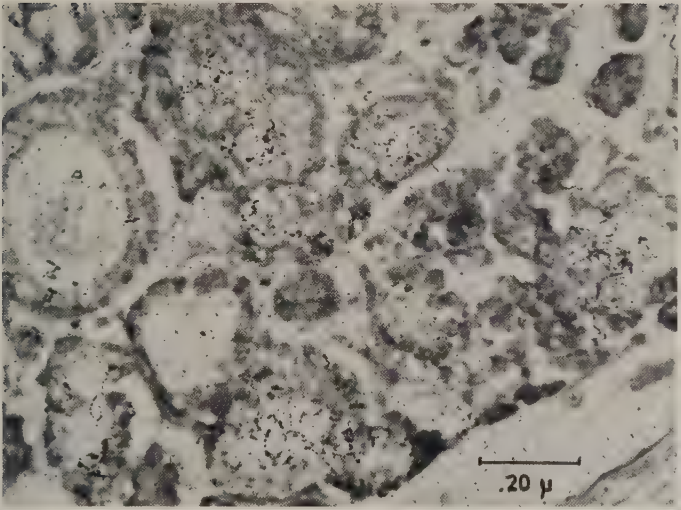

Fig. 1. Thyroid gland of rat. Electron-track autoradiography. Emulsion Ilford G5, thickness 40μ. Unna-Pappenheim staining. Several electron tracks can be seen emerging from colloid and, in a smaller number, from follicular epithelium.

organs (skin, genitourinary system, intestinal epithelium) of rats injected with radiosulphate [3, 4, 9, 10].

Dziewiatkowski [6] was able to isolate small amounts of ^{35}S-labeled cystine from the skin plus hair, the skeleton plus musculature and the internal organs of radiosulphate-injected rats. According to this author the labeled cystine was absorbed as such from the intestine, where the ^{35}S was incorporated into cystine by bacteria.

Since the thyroideal colloid does not seem to contain sulphurated polysaccharides [1, 11], an incorporation of ^{35}S into these compounds has to be excluded in our case.

On the basis of the present evidence, the only conclusion is warranted that the greatest part of ^{35}S taken up by thyroid gland is localized in the colloid. Experiments are in progress to identify the compound or the fraction in which ^{35}S is contained.

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CYTOLOGICAL FEATURES OF THE DEVELOPMENT OF THE EGGS OF *PARACENTROTUS LIVIDUS* REARED IN ARTIFICIAL SEA WATER DEVOID OF SULPHATE IONS

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In connection with work on the role of the different components of the sea water for the sea urchin development, Herbst [2,3] discovered that lack of sulphate ions causes a retardation of development of the archenteron which may gradually change into a pathological state of its cells. Moreover the dorsoventral organisation of the larva is suppressed and even an animalization of the larva may occur [12].

Lindahl [8,9] initiated a physiological analysis of the influence of lack of sulphate ions on the development. He suggested that the sulphate brings about a detoxication of phenols formed in the metabolism of the vegetal part of the sea urchin embryo.

By means of autoradiography Swedmark [13] demonstrated an uptake of S^{35}-labelled sulphate ions commencing at the blastula stage.

In this report some results with sea urchin embryos subjected to sulphate-free medium will be presented. The primary aim has been to test a conclusion drawn in a previous paper [7], viz. that the mucopolysaccharides, as demonstrated in the sea urchin eggs by the reaction of Hale [1, cf. 6], are esterified with sulphuric acid.

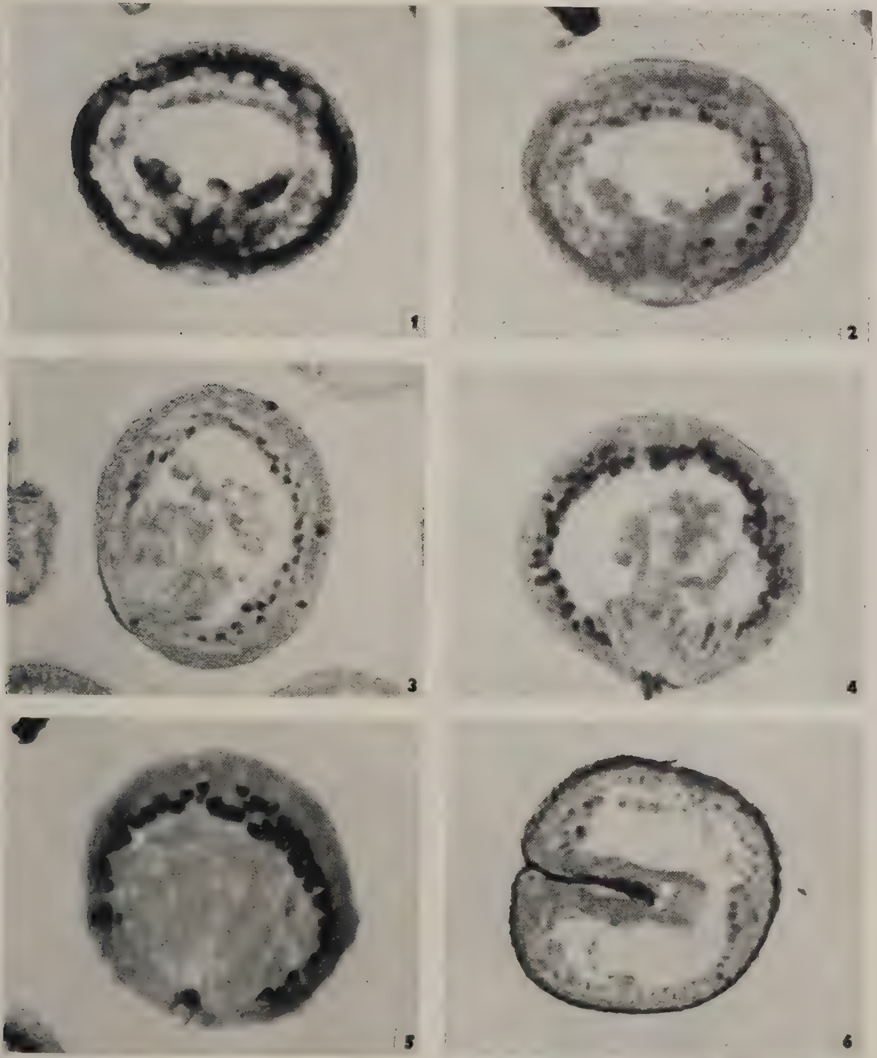
Methods. The experiments were carried out at the Laboratoire Arago, Banyuls-sur-Mer, France. The sea urchin *Paracentrotus lividus* served as material. The eggs were prepared in the customary way and fertilized with sperm freshly diluted from a sample of "dry sperm". The eggs and embryos were fixed in Carnoy fluid and the staining was made according to Hotchkiss [4] or Hale [1, cf. 6]. In most cases, however, the method of Ritter and Oleson [10] was employed, which combines the stainings according to Hotchkiss and Hale. By the use of an appropriate filter the red colour resulting from the Hotchkiss reaction may be eliminated, whereas the Hale positive structures appear dark.

In the preparation of sulphate-free medium the recipes given by Runnström [11] and Hörstadius [5] were followed. The salt concentration was adjusted to that prevailing in the sea water at Banyuls-sur-Mer.

Results.—The rate of development was lower in sulphate-free than in normal sea water. Moreover, in the former the larvae were less uniform with respect to the stage of development.

Until the blastula stage there is no difference with respect to the content and location of the acid mucopolysaccharides between larvae reared in normal and sulphate-free sea water. The blastocoele is narrower in the latter larvae, but contains acid mucopolysaccharides as in normal larvae.

Figs. 1–5 refer to the further development in sulphate-free sea water. It is evident that the Hale reaction is absent in the hyaline layer of the embryos. An animalization occurs in this medium; the entomesoderm remains rather undifferentiated. The α-gly-



Microphotographs of *Paracentrotus lividus* embryos, fixed in Carnoy's fluid and stained by Ritter and Oleson technique. Thickness of sections 6μ. Magn. 600 ×.
Figs. 1 to 5 represent embryos reared in artificial sea water, Fig. 6 represents an embryo reared in sea water. Fig. 1 photographed without filter, Figs. 2 to 6 with Ilford light-filter, No. 205, inserted. Without filter the stained α-glycol groups of carbohydrates and with filter the stained ester-bound sulphate groups and unsubstituted phosphate groups of nucleic acids appear black in photographs. Sulphate groups in exterior rim and archenteron, phosphate groups in nuclei. Figs. 1 and 2. Embryo 20 hours after fertilization. Fig. 3. Embryo 25 hours after fertilization. Fig. 4. Embryo 29 hours after fertilization. Figs. 5 and 6. Embryo 32 hours after fertilization.

col groups demonstrable with the Hotchkiss reaction are found in the cytoplasm, but not in the nuclei (Fig. 1). Invariably, the nuclei give a positive Feulgen reaction; *yet the coloration is deeper in the embryos exposed to sulphate-free sea water. Furthermore, the ectoderm nuclei stain with the Hale reagent, whereas this staining is absent in the entomesoderm.* As the figures show the staining of the ectoderm nuclei increases during the course of development, from 20 to 32 hours after fertilization. Apparently the staining commences at the inner margin and spreads gradually over the whole nuclei. The Hale reaction of the nuclei of control embryos (Fig. 6) is slight or wholly negative. After extraction with 5 per cent trichloracetic acid at 60°C for 8 minutes the nuclei neither stained with the Hale nor with the Feulgen method.

Discussion. Our data support the view that the Hale positive substance of the hyaline layer is constituted by mucopolysaccharides esterified with sulphuric acid [7]. These seem to resist the lack of sulphate in the cleavage and early blastula stage better than in the stages after hatching. This does not necessarily reflect a chemical change of the mucopolysaccharides. Possibly the turnover of the sulphate groups in the mucopolysaccharides increases during the development.

A striking result of this study is the strong Hale positive reaction of the ectodermic nuclei of the larvae reared in sulphate-free medium. There is a pronounced contrast to the negative behavior of the nuclei of the vegetal part. The staining is bound to nucleic acids as is proved by its disappearance upon treatment with hot trichloracetic acid. The staining of the nuclei with Hale's method very likely indicates that a splitting of bonds between proteins and nucleic acids has occurred, rendering phosphate groups available to the acetic iron reagent. It may thus be concluded that the ectodermic nuclei of the embryos reared in sulphate-free water contain nucleic acid rather than nucleoprotein. Staining with azur-eosin-methylene blue indicates that the nuclei contain both DNA and RNA. Further data are needed in order to decide whether the staining is due, not only to nuclear components, but also to RNA present in the immediate neighbourhood of the nucleus. In the mesentoderm the acid phosphate groups of the nucleic acids seem to be blocked by protein.

This investigation was supported by a grant from the Swedish Cancer Society to Professor J. Runnström under whose direction this research was carried out.

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METABOLIC ACTIVITY OF SALIVARY GLAND CHROMOSOMES IN DIPTERA

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THE giant chromosomes of Diptera can be regarded as highly polytene chromosomes in functional, fully differentiated cells, albeit of a somewhat unusual design. Their metabolism and mode of action deserves attention, not only for its intrinsic interest, but also for its possible relevance to the much wider question of the function of the cell nucleus in differentiated cells.

Previous attempts to solve the question of the metabolic activity of giant chromosomes in Diptera were concerned with the constitution of the chromosomes observed by staining techniques and enzymic digestion, and with the changes in the appearance

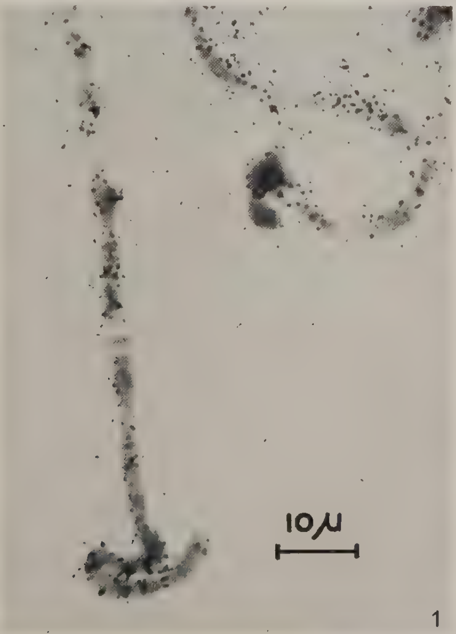

Fig. 1. Incorporation of ^{35}S-DL-methionine in chromosomes. Haematoxylin.

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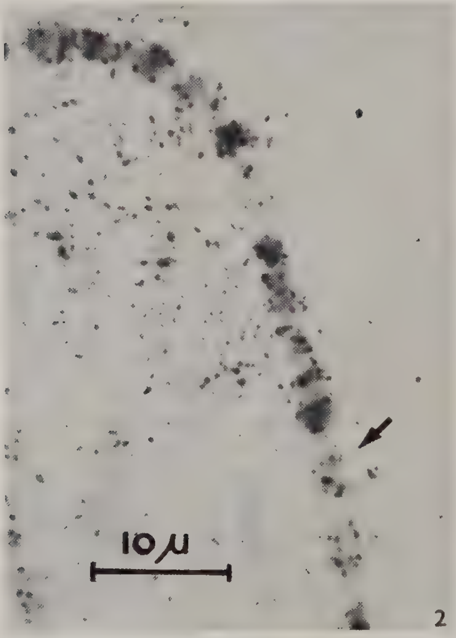

Fig. 2. Incorporation of [8-^{14}C]-adenine. The arrow indicates an unlabelled band. Haematoxylin.

of their bands and Balbiani's rings. Extensive lists of references, besides original investigations, can be found in recent papers by Kaufmann [5], Breuer and Pavan [2], Beermann [1], and Mechelke [6].

In the investigation presented here, the incorporation of ^{35}S-DL-methionine, [8-^{14}C]-adenine and $Na_2{}^{35}SO_4$ by salivary gland chromosomes of *Drosophila* was observed by means of autoradiographs.

Materials and methods. Larvae of *Drosophila melanogaster* (Oregon K.) were transferred to small vials containing approximately 1 g of food. Labelled compounds were dissolved in distilled water, and 0.1 ml of solution added to the food. ^{35}S-DL-methionine (approximately 50 μC per vial) and [8-^{14}C]-adenine (20 μC per vial) and ^{35}S as sodium-sulphate (100 μC per vial) were used. Twenty-four hours later, larvae shortly before pupation were selected, and their salivary glands dissected out and squashed in 45 per cent acetic acid: the coverslips were floated off in 10 per cent acetic acid. Some slides of the adenine experiment were treated with ribonuclease; the rest were washed and autoradiographs prepared by the stripping film technique [3].

Results and discussion.—Autoradiographs of cytoplasm and salivary gland chromosomes after 24 hours' treatment with ^{35}S-DL-methionine are shown in Fig. 1. The autoradiographs are not of even intensity along the chromosomes, the appearance of the straight part in Fig. 1 with a changing concentration being typical of

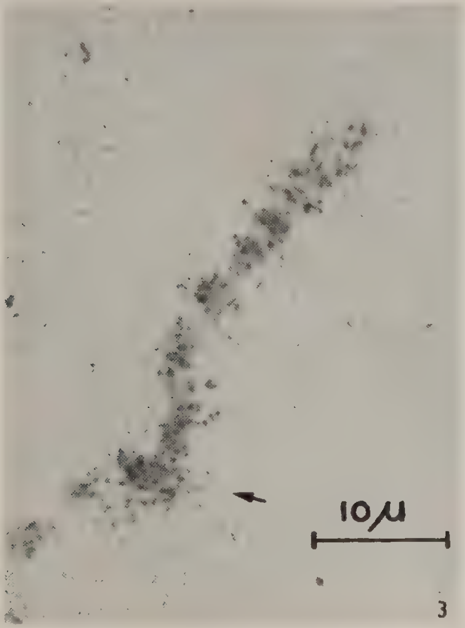

Fig. 3. Incorporation of [8-^{14}C]-adenine. The arrow indicates cytoplasm adhering to the chromosome. Haematoxylin.

these preparations. These autoradiographs are not sufficiently strong to decide the relative concentration in small portions, such as the bands of the chromosomes.

Good autoradiographs are obtained after 24 hours' treatment with [8-^{14}C]-adenine. Fig. 2 shows that the grains are mainly above bands of chromosomes which are free from cytoplasm, the cytoplasm being well labelled. Some bands are unlabelled (arrow in Fig. 2). Remnants of cytoplasm adhering to salivary gland chromosomes (Fig. 3) can be seen clearly under phase contrast, and the chromosomes were carefully examined before being stained and photographed.

After treatment with ribonuclease, the preparations show no autoradiograph, indicating that most or all of the ^{14}C was incorporated into ribose nucleic acid. This finding confirms previous work on the presence of RNA in chromosomes [4, 7]. The deoxyribonucleic acid of the bands is either completely unlabelled or too weak to give autoradiographs even after 60 days' exposure. It is of interest to note that at least the metabolically active ribose nucleic acid of the chromosomes is located in the bands.

No autoradiographs of salivary gland chromosomes or cytoplasm were obtained after application of ^{35}S in the form of sulphate, which could be expected to be incorporated in sulphated mucopolysaccharides. This observation makes it certain that the autoradiographs observed after application of ^{35}S-labelled DL-methionine are due to incorporation into proteins, and not to incorporation of the radioactive sulphur in sulphated compounds.

The incorporation of methionine and adenine may be allied with growth of the chromosomes or with metabolic activity of their RNA and proteins. The uneven uptake along the chromosomes, the lack of RNA labelling of some bands, and the fact that the DNA is not labelled suggest that the uptake is not due to growth, and we conclude that the incorporations we have observed indicate metabolic activity of the proteins and of the ribonucleic acid associated with the bands. The method described above thus offers a possibility of investigating the metabolism of salivary gland chromosomes, whose metabolic function is at present unknown, and of determining the function of single bands or groups of bands in these chromosomes.

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ELECTRONMICROGRAPHIC STUDY OF FLAGELLATED EPITHELIA IN MADREPORARIAN CORALS

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Ciliary currents are of major importance in the feeding of corals but knowledge regarding the nature of the flagellated epithelia in these animals is fragmentary. Observations were carried out by electron micrography of ultrathin sections in *Astrangia danae* Milne Edwards and Haime and *Mussa angulosa* Pallas.

The columnar supporting cells, the main components of the flagellated epithelia in the corals, are between 20 and 80 μ long and 0.5 to 5.0 μ in diameter with the nuclei located in the middle. The cytoplasm is filled with locular inclusions of all sizes as seen in Fig. 1. Each cell bears a single flagellum that arises in a pitlike depression the rim of which is raised to form a collar process (Fig. 2).

The appearance of the collar processes differs considerably in the two species studied. In *A. danae*, they are simple and lacking in appendages whereas in *M. angu-*

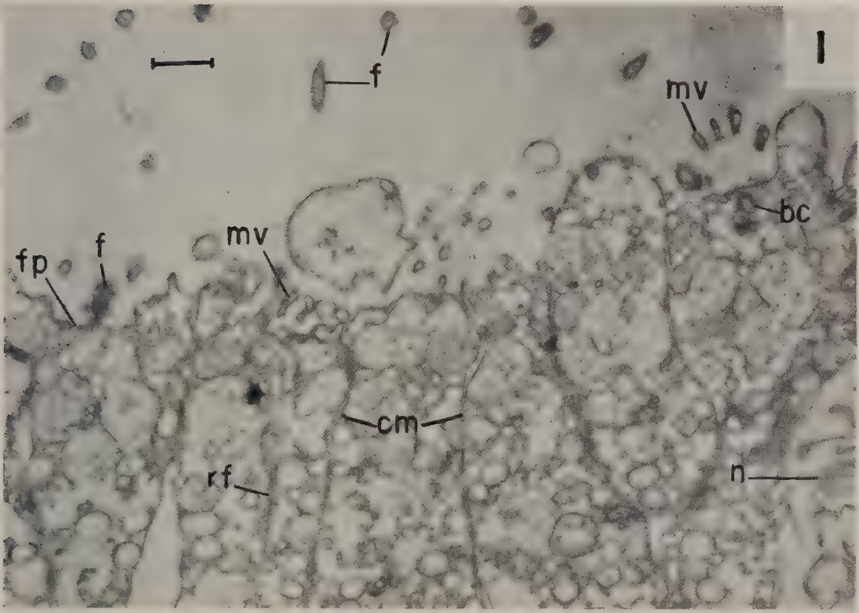
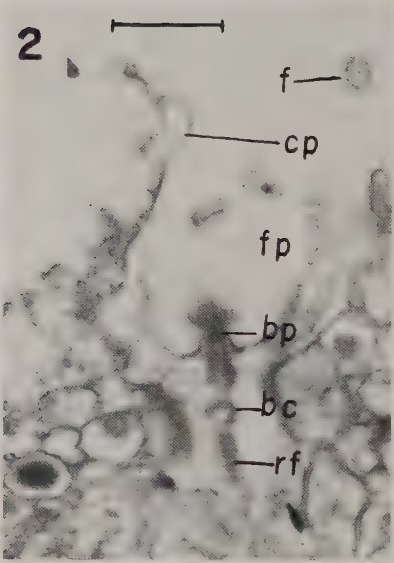


Fig. 1. Stomodeum of *M. angulosa* showing superficial region of columnar supporting cells. Note continuous cell membranes and flagellum arising from cell on the left side. Magnification × 12,000.

Abbreviations: bc, basal corpuscles; *bp*, basal plate; *cm*, cell membrane; *cp*, collar process; *f*, flagellae; *fm*, flagellar membrane; *fp*, flagellar pit; *lf*, longitudinal filament; *mv*, microvilli; *n*, nematocyst; *rf*, root filament.

losa they are complex, giving rise to between nine and twelve evenly spaced microvilli as shown in Fig. 5. Microvilli not associated with collar processes are also found. These are about 2 μ long by 100 mμ in diameter, and are more numerous on the cells of the septal filaments than on the epidermal cells of the stomodeum and oral disk. At the present time it has not been possible to decide whether the microvilli are transient pseudopodial outgrowths or permanent cellular appendages.

The flagellae are about 250 mμ in diameter and about 50 μ long. Nine pairs of longitudinal fibrils are arranged in a circle around a central pair as shown in Fig. 3. Each fibril is about 200 Å in diameter and is enclosed by a limiting membrane giving the appearance of a very small tubule. The flagellae possess basal plates through which they are connected to the basal corpuscle by a short fibrous segment about 300 mμ long. The basal corpuscles are about 300 mμ in diameter and give rise to a single unbranched root filament possessing cross striations with a period of 670 Å (Fig. 4).



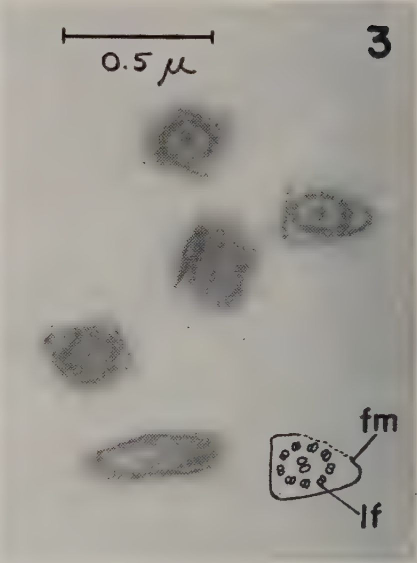


Fig. 2. Epidermal cell from oral disk of *A. danae* with the root of the flagellum. The root filament has cross striations. Magnification × 22,000.

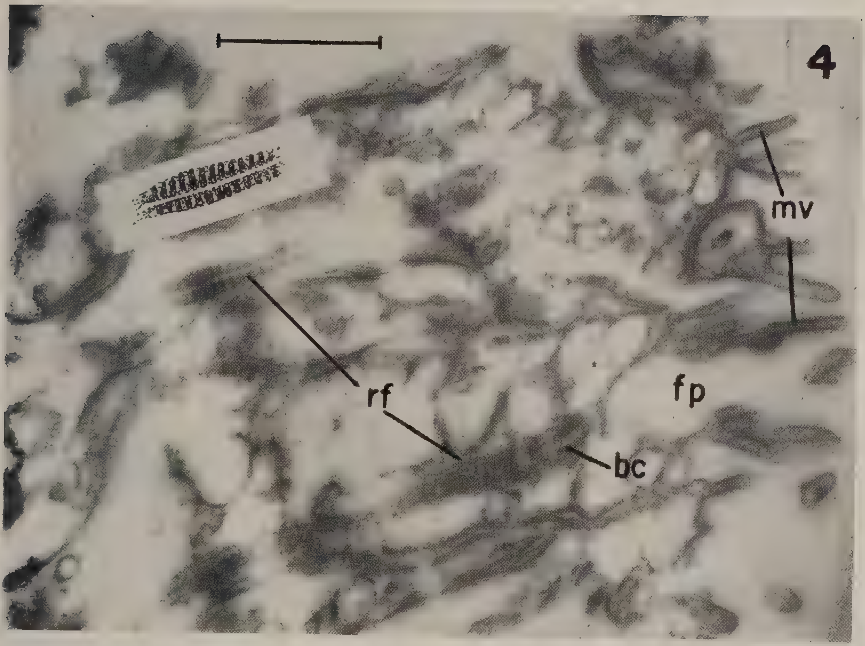
Fig. 3. Cross section through epidermal flagellae of *A. danae*. The inset shows the fibrils in greater detail. Magnification × 60,000.

The fine structure of coral flagellae closely resembles that of analogous organs described in brown algae by Manton [2] and her co-workers [3], in lamellibranchs, frog and mouse by Fawcett and Porter [1] and in rat spermatozoa by Watson [5]. Although no distinction is possible on the level of fine structure, it is clear that the whip organs of corals are flagellae, not cilia, since there is only one per cell.

At the magnifications employed in this study, all cells of the coral species and tissues examined so far were found to be mononucleated units invested by cell membranes which show no evidence for the existence of protoplasmic bridges. Our observations therefore do not support Matthai's [4] conclusion that the epithelia of madreporarian corals are syncytial structures.

Fig. 4. Ciliated tract in septal filaments of *M. angulosa*. Longitudinal sections of two cells demonstrate the cross striations of the root filament. The inset shows the striations in more detail. The microvilli on the lower cell arise from the edge of the flagellar pit. Magnification × 31,000.

Fig. 5. Tangential section through same region as in Fig. 4 showing the relationship of the microvilli to the flagellae. Magnification × 19,900.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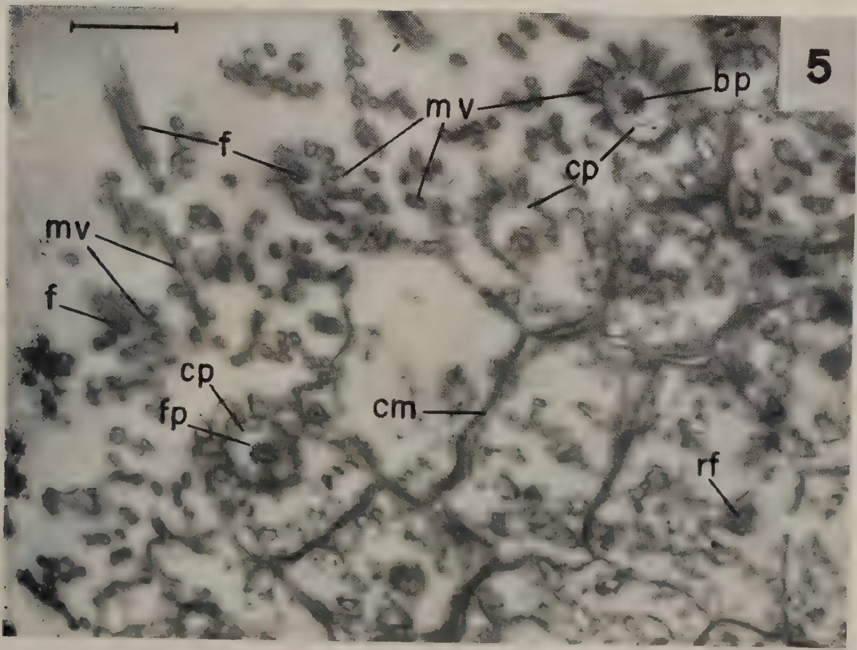
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LIPID PEROXIDE FORMATION IN REGENERATING RAT LIVER[1]

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Homogenates of liver, kidney, and brain produce lipid peroxides on aerobic incubation, and these can be estimated by the thiobarbituric (TBA) reagent [1, 4]. Preparations of bone marrow do not form peroxides on incubation [2, 5] although they contain the requisite lipids. Moreover, bone marrow suspensions added to liver homogenates inhibit peroxide formation in the latter [2]. These facts indicate that marrow contains antioxidants. Lipid peroxides formed by exposing pure methyl linolenate to ultraviolet radiation inhibit the growth of *E. coli* [6] and the division of the eggs of the annelid *Chaetopterus* [8]. Since oxidized lipids inhibit cell division, dividing cells may possess some mechanism to prevent the accumulation of lipid peroxides. To test this possibility peroxide formation was estimated in rat liver during periods of high and low mitotic activity following partial hepatectomy.

Methods. The left and median lobes of the liver were removed from Wistar rats under ether anesthesia. The normal tissue removed provided control values. The animals were killed 10, 48, or 144 hours later and the regenerating liver was taken for analysis. These times correspond respectively to a period of increase in cell size with low mitotic rate, a period of active mitosis, and a later period of low mitotic activity. A 1:15 homogenate was made in $0.07 M$ Na-K phosphate buffer, pH 7.4, and duplicate flasks containing 2 ml of homogenate were incubated at 37°C with shaking for 2 hours with and without 0.2 mg/ml of ascorbic acid. Ascorbic acid catalyzes fatty acid peroxide production in tissues [1], and its use gave a measure of the amount of peroxide which the tissue could produce under the experimental conditions. At the end of the incubation period 2.0 ml of 20 per cent trichloroacetic acid and 4.0 ml of 0.67 per cent TBA were added. The mixture was placed in a boiling water bath

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[1] Aided by a grant from the Atomic Energy Commission.

TABLE I

TBA values of normal and regenerating liver. Figures give mean values and standard deviation for TBA optical density as described under Methods. Measurements on 45 normal rat livers and 14, 16, and 16 regenerating livers at 10, 48, and 144 hours respectively.

Normal liver			Regenerating liver			
Ascorbic acid	No ascorbic acid	Difference	Time after operation, hrs.	Ascorbic acid	No ascorbic acid	Difference
0.42 ± .13	0.21 ± .08	0.21 ± .08	10	0.62 ± .10	0.21 ± .12	0.41 ± .11
			48	0.27 ± .07	0.11 ± .04	0.16 ± .05
			144	0.57 ± .08	0.24 ± .05	0.33 ± .07

for 15 minutes, centrifuged, and the optical density of the supernatant measured at the absorption maximum. The optical density values in Table I and Fig. 1 are for supernatants diluted 1:4.

Results and discussion.—The mean TBA values for normal and regenerating liver are shown in Table I. Columns 3 and 7 give the increment in peroxide formation as a result of ascorbic acid catalysis. Regenerating liver taken 48 hours after partial hepatectomy when mitosis is maximal showed minimal peroxide values and a minimal increment in the presence of ascorbic acid. The peroxide values at this time are also lower than the corresponding values for normal liver. Differences between normal and regenerating liver in increments resulting from ascorbic acid catalysis (columns 3 and 7) were all significant at the 1–2 per cent level.

The absorption spectra of the TBA reaction for normal and 10-hour regenerating liver from the same rat with and without ascorbic acid are given in Fig. 1. The curves correspond to those given by oxidized linolenic and linoleic acid ([7], unpublished results).

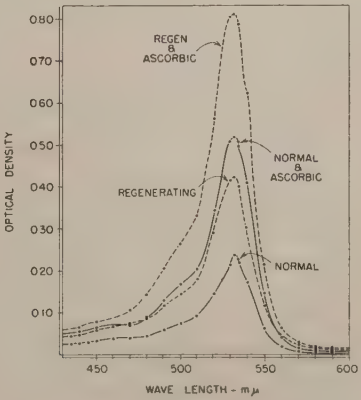

Fig. 1. TBA absorption spectra of normal and regenerating liver after 10 hours. All curves corrected for reagent blank.

The low TBA values after 48 hours regeneration and at the height of mitotic activity suggest increased antioxidant activity which inhibits the effect of added ascorbic acid and endogenous catalysts. The effect is particularly striking because of the increase of unsaturated fatty acids in regenerating liver [3] which may form peroxides. (Arachidonic acid does not enter into these results as it gives no color with TBA after incubation [7].) Antioxidant activity appears to be present at 144 hours regeneration also, since the value for liver incubated without ascorbic acid is approximately normal even though the content of unsaturated fatty acids is increased [3]. The extent of the increase in TBA value on incubation in a particular instance will reflect the unsaturated fatty acid content, catalysts, and antioxidant activity. In addition to the effects of specific antioxidants the antioxidant activity may include binding and complex formation between lipids and other substances.

The correlation between low TBA values and mitosis of regenerating liver is in line with previous findings that peroxides are not formed in bone marrow where cell division is taking place. In normal liver the mitotic rate is low and remains low during the early stages of regeneration when the TBA value (no added ascorbic acid) is normal. Active mitosis then follows during a period when TBA values are decreased. Later (144 hours) when the mitotic rate is again low the TBA values have increased. In view of the inhibitory effect of fatty acid peroxides on cell division the present results raise the question whether antioxidants and/or lipid peroxides are involved in the initiation and cessation of mitosis in regenerating liver.

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THE OCCURRENCE OF MAST CELLS IN THE MOUSE VAGINA IN PROLONGED OESTROGENIC TREATMENT

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The metachromatic properties of loose connective tissue has been studied by several investigators. In 1934 Sylvia Bensley [1] found that growth and metachromasia were correlated during the proliferative phase of the human endometrium. When the stroma had completed its growth, practically no metachromatic substance was seen, except in the margin of the mucous membrane. This correlation between growth and metachromasia was later confirmed both in humans and in animals by Holmgren [2, 3] and Sylvén [6]. According to Layton [4] it is probable that radiosulphate is incorporated in ester sulphates of healing wounds.

In this connection it is interesting to note the behaviour of mast cells during growth. In recent quantitative investigations [7, 8] it was shown that a moderate single dose of oestrogenic hormone, stimulating growth of uterus and vagina, significantly decreased the number of mast cells in the uterus and vagina compared to untreated controls.

In 1952 Salvi [5] found an increased number of mast cells in haemorrhagic metropathy of humans. It was therefore considered of some interest to investigate if prolonged oestrogenic treatment stimulating growth of the vaginal tissues might influence the incidence of mast cells in animals in a similar way as in humans.

TABLE I

Experimental treatment of animals.

Group	Number of animals	Pretreatment	Treatment on day														
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
C_p	3	h	s	–	s	–	s	–	s	–	s	–	s	–	s	–	+
O_p	4	h	o	–	o	–	o	–	o	–	o	–	o	–	o	–	+

h = hysterectomy and bilateral oophorectomy two weeks before treatment.
s = 0.2 ml sesam oil subcutaneously.
o = 0.2 ml (20 I.b.E.) oestradiol subcutaneously.
+ = animals sacrificed.

TABLE II

Mast cells of the vagina in prolonged oestrogenic treatment.

Group	$M \pm \varepsilon M$	σ	n
C_p	130 ± 3.0	9.0	9
O_p	199 ± 6.4	22.1	12

Experimental.—Seven white mice, weighing about 20 g, were used. In ether anaesthesia a total hysterectomy was made in connection with bilateral oophorectomy. Three animals served as controls (group C_p). The remainder (group O_p) received prolonged oestrogenic treatment. The treatment of the two groups is indicated in Table I. The animals were killed with ether, and vaginal transverse sections just below the external os of the uterus were sampled.

The pieces of tissues were fixed in excess of methanol and embedded in paraffin. Three complete 5μ sections from the cranial part of each vaginal block were stained with 0.1 per cent toluidine blue in 30 per cent methanol solution. For further details a previous investigation is referred to (Westin [7]). The total number of metachromatic mast cells per transverse section area was counted using about 480 × magnification.

Results and discussion.—The mast cells of the vagina took an intense dark metachromatic staining in both groups. Separate granules of the mast cells could not be distinguished. In both groups the mast cells were most abundant in the subepithelial connective tissue.

The results of the quantitative analysis are indicated in Table II. Prolonged oestrogenic treatment (group O_p) significantly increased the number of mast cells compared to the control group (C_p).

The influence of prolonged oestrogenic treatment on the occurrence of mast cells in the mouse vagina is correlated to the results obtained in human metropathy [5].

It may be said that growth induced by prolonged oestrogenic treatment was accompanied by a significant increase in the number of mast cells of the vagina. The reason why this occurs is not clear. One possible explanation may be that the appearance of mast cells during growth is secondary to an increased vascularization.

Summary.—The occurrence of mast cells in the mouse vagina was studied after hysterectomy and bilateral oophorectomy. Growth induced by prolonged oestrogenic treatment was accompanied by a significant increase in the number of mast cells compared to untreated controls.

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DEMONSTRATION OF A- AND B-CELLS OF THE ISLETS OF LANGERHANS BY A MODIFICATION OF MANN'S TECHNIQUE

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The chief point in which the method discussed below differs from Mann's original methyl blue–eosin stain ([1], § 728) is the oxidation with a solution of sulphuric potassium permanganate. This treatment prevents the exocrine tissue from becoming red and the also red-staining A-cells are distinguishable with certainty. Another difference is that after staining the sections are treated with solutions in which absolute alcohol is the basis. Hence, risk of differentiation during after-staining is avoided and the procedure somewhat shortened.

Particular attention has been paid to the effect of the pH of the dye solution on staining and to the value of light green as a counter-stain.

The method has been elaborated for the quantitative study of the different cell types in the pancreatic islets of Langerhans with special reference to the hedgehog (*Erinaceus europaeus* L.). In the hedgehog the islets of Langerhans are often very diffuse and the cells on the edges of the islets are quite close to the cells of the exocrine tissue. Therefore it is necessary for the cells of the exocrine tissue to be stained differently from the islet cells.

The course of staining.—As fixative Bouin's solution ([1], § 305) was used (8–24 hours). The sections were cut at 5 microns. In transference to water the sections were passed quickly through the alcohol series because this seems to improve their stainability. (1) Picric acid is washed out in running water. (2) Oxidation with a mixture of potassium permanganate and sulphuric acid for 1–2 mins. (3) Bleaching in a 3–5 per cent solution of sodium bisulphite. (4) Rinsing in running tapwater over 3 mins. and then quickly in distilled water. (5) Staining 2–3 days. (6) Differentiation until the dark colour of the sections has just vanished and changed to a clear brownish red. If light green is used the colour remains a little greyish. Differentiation is easy and can be checked macroscopically. (7) Rinsing in acetic alcohol (3 cc of acetic acid, glac., in 100 cc absolute alcohol) until the dark macroscopic colour has returned (in about 3–5 mins.). (8) Passing quickly through absolute alcohols. Xylene and mounting in Canada balsam or "Caedax".

Formulae of solutions.—1. Oxidizing solution: Sodium permanganate, satur., 1; sulphuric acid, 5 per cent solution, 1; water, (6–7). *2.* Staining solution ([1], § 728): Methyl blue, 1 per cent solution, 35 cc; eosin (yellowish), 1 per cent solution, 45 cc; distilled water, 100 cc. Acetic acid is used to increase the acidity of the staining solution.

Differentiating solution.—To 100 cc of a saturated solution of orange G in absolute alcohol is added 3–3.5 cc of a solution made up of 0.3 cc of 30 per cent sodium hydroxide solution and 10 cc of absolute alcohol. The light green solution contained, when used, 50–70 cc absolute alcohol saturated with light green (yellowish). The saturated solution is made up to 100 cc with absolute alcohol after filtration and then added to the above-mentioned alkaline orange G mixture.

The result of staining greatly depends on the pH of the dye solution. The useful pH range is around 4.0–5.4. The latter reveals the acidity of solution, which is made up according to the directions. The exocrine tissue, and especially its granules, are stained dark. The colour is influenced by the pH and varies from dark brown (at pH 4.6–5.4) to pure blue (at pH under 4.6). The plasma of the B-cells remains virtually colourless. The small granules of the A-cells are stained red, the red becoming more predominantly violet as the solution becomes more acid. Only the erythrocytes of the blood also stain red and they are clearly distinguished from the A-cells. The nuclei are stained brownish black at pH values over 4.6, and dark reddish violet just below this pH value. If the pH falls below 4.0 the latter colour becomes so intense everywhere that the stain is unfit for use.

It is only necessary to increase the acidity when the exocrine tissue is poor in granules, for it improves the contrast between the islets and the now dark blue exocrine tissue. For the same reason, photography succeeds better when the staining is made at a lower pH.

Light green is successful as a counter-stain only if it is added to the differentiating solution. The use of light green makes the staining a little uncertain, apparently owing to the readiness of this stain to differentiate in the alcohol series, where the sections are treated after the proper differentiation. It must be granted, however, that if the use of light green succeeds, the staining is one of the best possible. The exocrine tissue, especially its plasma, is now a clear green. The plasma of the islet cells also shows a faint green colour.

As a whole the staining is quick and easy and gives quite satisfactory results. The separate A-cells are distinguishable without difficulty, as are the small croups of B-cells. The identification of isolated B-cells is, on the other hand, somewhat subjective.

The methyl blue.—The methyl blue used in these experiments was provided by two factories, Merck and Gurr. Of these, only Merck's product proved suitable. The difference between them is brought out when comparing the colour of weak aqueous solutions in an acid and in a basic solution. The mixture made of Gurr's methyl blue remained blue quite independently of the pH of the medium. On the other hand, Merck's methyl blue solution was blue in an acid medium and red in a basic medium, the changing point occurring almost exactly at pH 7.0, where a weak solution became almost colourless. It may be mentioned that the alkaline red solution, after standing for some time, entirely lost its colour. This was, however, reverted to blue, when the solution was again made acid. A corresponding vanishing of the colour has not been observed in the preparations, although some of these are one year old.

The staining procedure has evolved from investigations on the hibernation of the hedgehog, which have been carried out under the direction of Prof. Paavo Suomalainen at the Zoological Laboratory, the University of Helsinki.

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THE FILAMENTOUS FINE STRUCTURE OF STRIATED MUSCLE AT DIFFERENT STAGES OF SHORTENING[1]

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Since it is now generally believed that muscular contraction and relaxation involve a reversible interaction of the proteins actin and myosin, the identification of these two components in high resolution electron micrographs would greatly advance our understanding of the contractile mechanism. Previous studies of the structure of striated muscle with this aim in mind have led to several models, which are in certain respects contradictory. According to one view, only a single set of filaments extends continuously through all levels of the sarcomeres [1, 2, 6, 16]. Huxley [9] and Hanson and Huxley [3], however, believe that two sets of filaments exist, a thick (myosin) and a thin (actin) set, and that these interdigitate in the *A* band.

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It is probably safe to state that in most of the previously published work the muscle was not maintained at rest length during fixation (which itself causes shortening). The work described in the present paper was made possible by taking great care to hold the muscle fibers at known lengths throughout the preparative procedures which were similar in general to those previously described [7, 8]. Rabbit psoas and chick leg muscle were fixed in the fresh state, after glycerination, or after glycerinization followed by plasticizing [17, 18]. From these studies there has emerged a new model and a suggestion for a mechanism of contraction based on interaction of actin and myosin at the macromolecular level.

Longitudinal sections of fibrils fixed at either rest or equilibrium lengths (the *I* band is not less than $\frac{1}{2}$ the length of the *A* band) are characterized by the following features (see Figs. 1 and 2): In the *H* band the filaments are thick (ca. 120 Å) and are spaced 200–300 Å apart. In the *A* band proper (on either side of the *H* band) the filaments are thinner (60–70 Å) apart, and are approximately *twice* as numerous as the thick filaments in the *H* band. Also the density of the *A* band interfilamentous region is greater than in the *H* band. The multiple (ca. 1.7) ratio of thin to thick filaments in the *A* versus *H* bands may be explained by supposing that several thin filaments aggregate to form one thick filament. Convergence of pairs of thin filaments to form single thick filaments has been repeatedly observed in favorable sections (see Fig. 3). The *I* bands present a somewhat disorganized array of thin filaments with a very low interfilamentous density. The *Z* membranes (in the center of the *I* bands), however, are traversed by the same number and type of filaments as are present in the *A* band proper (Figs. 1 and 2). Therefore one may assume continuity of thin filaments from the *Z* membrane through both *A* and *I* bands up to the *H* band, where a number of them associate to form the thicker filaments.

The filamentous structure of myofibrils which have been allowed to shorten to about 65 per cent of rest length is strikingly different from that described above (see Fig. 4). In such fibrils the entire *A* band (*A* band proper as well as *H* band) now contains only thick filaments. But at this length, thin filaments can still be seen penetrating the *Z* membranes in the center of the much shortened *I* bands. Further decrease in sarcomere length results in the disappearance of the *I* bands; now thick filaments transverse the entire sarcomere.

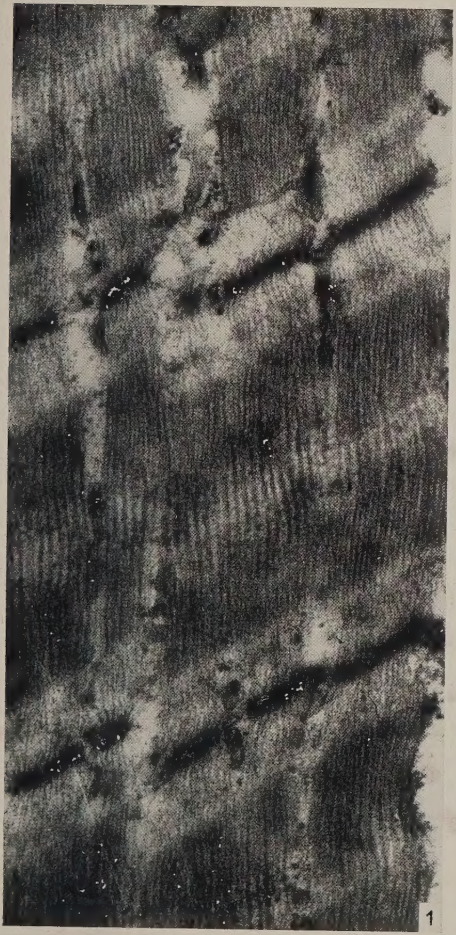
It is clear that on shortening some mechanism causes adjacent thin filaments to aggregate in the form of thick filaments. As an interpretation the following is at present preferred. Convincing evidence has been given to support the view that myosin occurs primarily in the *A* band and is reponsible for the major fraction of the positive birefringence of that region [4, 5]. The fibrous lattice of the myofibril is presumably due largely to *F* actin which may be identified with the thin filaments. If myosin in the muscle fibril occurs in the form similar to that in solution after extraction, it would consist of a dispersion of macromolecules having dimensions of about 20 × 2000 Å [15]. We should like to suggest that these myosin macromolecules are present between the thin filaments in the *A* band and are chiefly responsible for the interfilamentous density noted in that region.

When shortening occurs, with attendant splitting of ATP, actin and myosin unite to form a firm complex. We suggest that this is made possible by a deposition of the long thin molecular units of myosin upon the thin filaments of actin. If these two macromolecular types fit together in a stereochemically favorable fashion the complex may form a compound super-coil or rod, which is reflected by the transformation of thin filaments into the thicker ones in the *A* band. Because of such complementarity and relative perfection of fitting, the tendency will be to form a coiled-coil which in turn would involve more and more of the surfaces of the actin (thin) filaments until they are completely associated with myosin, i.e. the actin filaments have been drawn entirely into the *A* band and the *I* band has disappeared. (Several workers have recently demonstrated that on shortening it is the *I* band primarily which changes in length [12, 13].) Such a mechanism avoids invoking long range forces but proposes a macromolecular complementarity of structure of actin and myosin as the source of spontaneous and rapid combination of these two molecular species when the chemical environment is appropriately changed (splitting of ATP, etc.) upon stimulation. It is possible that the "hollow" appearance of the thick filaments noted by Huxley [9] and Hodge [6] may reflect the compound helical coiling of the actomyosin complex. On relaxation the new supply of ATP combines with one or both of the fibrous protein partners, reducing their affinity and causing them to dissociate and thus uncoil. The nature of the thick filaments in the *H* band itself is not clear; according to several workers [3, 4, 9, 14] they do differ from their counterparts in the *A* band proper.

In explanation of the different conclusions and model arrived at by Huxley and Hanson it may tentatively be suggested that the thin filaments seen by them in transverse sections to occur at trigonal positions relative to the thick filaments may have represented the same structures, cut in a very particular plane, which have been observed by various investigators, especially Hodge [6] and which have been called lateral or connecting bridges. Huxley's [10, 11] earlier x-ray evidence for two sets of filaments does not require that the two sets occur in the sarcomere *at the same level*; it could reflect the presence of thick and thin filaments observed in the present work rather than the interdigitating filaments hypothesized by Huxley and Hanson.

It is fully realized that the above suggestions do not explain all the facts which have been brought out by electron microscope and light microscope studies of muscle structure. They are primarily concerned with a clarification of the filamentous structure of the resting and shortened myofibril, and with putting forward, as an explanation of the contractile process, an hypothesis based upon the properties of elongate macromolecules of myosin in their reversible association with the actin component. Details of the experiments will be described in due course.

Fig. 1. Section of chick leg muscle fixed at equilibrium length. × 50,000.
Fig. 2. Equilibrium length chick leg muscle. × 100,000.
Fig. 3. *A* band–*H* band junction of rabbit psoas fixed at rest length. × 75,000.
Fig. 4. Section of chick leg muscle shortened to about 65 per cent of rest length. × 50,000.



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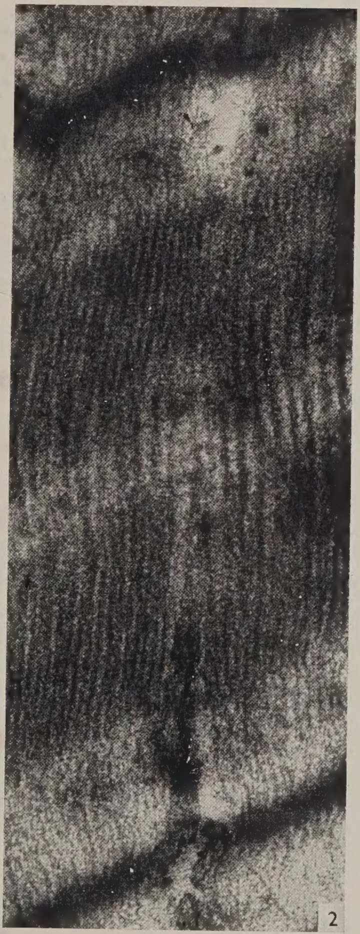

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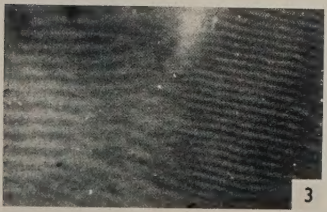

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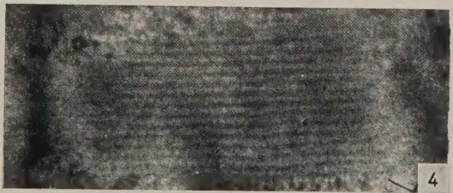

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ASPECT AU MICROSCOPE ELECTRONIQUE DE CERTAINES SYNAPSES INTERNEURONALES AXO-SOMATIQUES[1]

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Les méthodes classiques de l'histologie du système nerveux, et en particulier l'imprégnation argentique, montrent que les fibres nerveuses se terminent par un renflement appelé « bouton terminal » s'il est petit, « massue » ou « club » s'il dépasse 3 microns de diamètre. Le microscope électronique a permis d'observer la structure de ces boutons. Ainsi, les terminaisons nerveuses sur les cellules auditives sont décrites par Engström et Sjöstrand [3] comme des formations arrondies, limitées par une fine membrane qui les sépare complètement de la cellule sensorielle, et dans l'intérieur desquelles il est possible de distinguer une zone à mitochondries séparée de la membrane synaptique par une zone à granulations. Un aspect analogue a été trouvé par De Robertis [2] dans le cas des synapses interneuronales axo-somatiques.

Grâce à une méthode améliorée d'imprégnation argentique, Weber [5] a pu observer au delà de certains boutons terminaux un « appareil métaterminal », constitué

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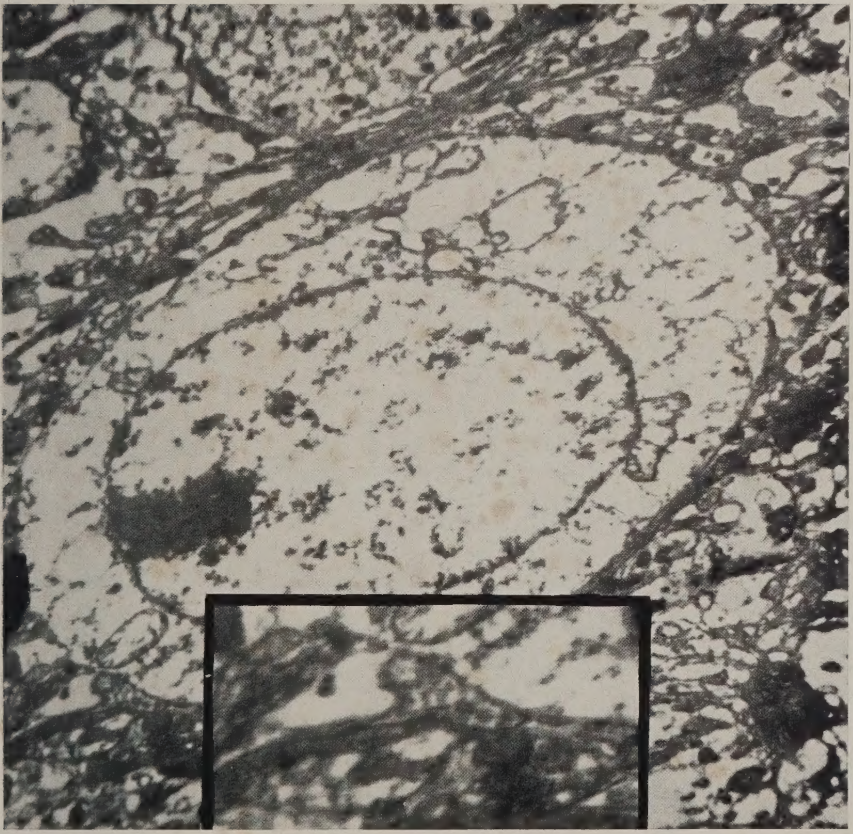

Fig. 1. Cellule de Purkinje dans une coupe de cortex cérébelleux de souris. *En bas, à gauche*, terminaison nerveuse au contact de la membrane cellulaire; au voisinage de cette terminaison, deux renflements nerveux préterminaux. Grossissement 10000 ×. *Dans le carton inséré au bas de la figure*, même image, grossissement 20000 ×. Fixation à pH = 6,4 donnant au cytoplasme et au noyau un aspect particulier.

par un filament extrèmement fin, long de quelques microns et d'un diamètre de $0{,}1\mu$ environ, donc à la limite du pouvoir résolvant d'un microscope à lumière. Il est possible aussi de voir ces formations au microscope électronique.

Le matériel utilisé pour ces observations est constitué par du cervelet de souris. Sur l'animal anesthésié avec une dose minime de Nembutal, la boîte cranienne est ouverte, et la surface du cervelet immédiatement recouverte de fixateur de Palade (acide osmique tamponné); de petits fragments de cortex cérébelleux sont prélevés avec une lame de rasoir, et plongés dans le même fixateur pendant deux heures, puis inclus dans le méthacrylate. Les coupes au $\frac{1}{40}$ de μ sont faites au microtome Porter-Blum de Sorvall, et observées au microscope électronique Trüb-Täuber muni d'un diaphragme de contraste.

Les résultats obtenus peuvent être vus, en partie au moins, sur la figure 1, qui représente une cellule de Purkinje du cervelet. Au pôle inférieur de cette cellule, on voit s'accoler à la membrane cellulaire, par une extrémité évasée, un mince filament nerveux; l'accolement est direct, sans interposition d'aucune formation névroglique ou autre. Le filament et son expansion terminale sont limités par une fine membrane continue; le neuroplasme qu'ils contiennent présente de petites vésicules et des granulations, analogues à celles que décrivent Engström et Sjöstrand [3] et De Robertis [2]; on y remarque aussi un petit faisceau de neuroprotofibrilles, ressemblant à ce qu'a montré Fernández-Morán [4].

On ne voit pas de mitochondries dans cette terminaison nerveuse, que sa forme et ses dimensions permettent de considérer comme un appareil métaterminal. Par contre, dans d'autres renflements nerveux un peu plus gros situés dans le voisinage immédiat du précédent, des mitochondries sont visibles; mais ces renflements ne sont pas les véritables terminaisons, puisqu'un prolongement sans mitochondries leur fait suite, qui est retrouvé dans les coupes ultérieures de la même série.

En conclusion, les aspects observés amènent à penser que la terminaison nerveuse avec appareil métaterminal diffère du bouton terminal simple en ce que la zone la plus distale de celui-ci (celle qui contient des vésicules et des granulations, mais pas de mitochondries) est devenue beaucoup plus longue et plus mince.

On peut encore rappeler que les molécules protéiques qui entrent dans la constitution de la membrane de l'appareil métaterminal sont nettement orientées parallèlement à l'axe de cette formation, puisqu'après imprégnation argentique on y observe un fort dichroïsme [1]. Si l'on admet de plus, comme le pense De Robertis [2], que les granulations et vésicules synaptiques sont associées aux précurseurs des médiateurs chimiques, on trouve dans la synapse les ultrastructures en rapport avec son fonctionnement : membrane protéique faite de molécules orientées pour la conduction électrique, et médiateurs chimiques.

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